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14. ABSTRACT

To determine if AR gene silencing in prostate cancer cells via RNA interference mechanism leads to disruption of androgen-independent progression. We generated a recombinant AAV for long-term expression of a hairpin-structured AR siRNA in vivo. Then we determined the essential role of the androgen receptor in androgen-independent growth of prostate cancer. We demonstrated that knocking down AR expression abolished tumor growth and blocked androgen-independent transition in LNCaP and LAPC-4 cell-derived xenografts. In addition, we observed that AR silencing resulted in a significant decrease of androgen-independent tumor growth in C4-2 cell-derived xenografts in castrated mice. We also demonstrated that knocking down AR expression by systemic delivery of the AAV particle eradicated CWR22RV1 androgen-independent xenograft tumors but not AR-null PC-3 xenograft tumors. We analyzed the mechanisms for AR siRNA-induced cell death and identified the anti-apoptotic proteins SGK1 and Bcl-xL, as the downstream effecters of AR-mediated survival pathway. Most of these results were published (Appendix II-VI).

15. SUBJECT TERMS
Prostate Cancer Xenograft; RNA Interference, siRNA, Androgen Receptor; Adeno-Associated Virus; Prostate Cancer Cell Line; Cell Culture; Apoptosis

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Table of Contents

Page

Final Report for the Project W81XWH-04-1-0214

INHIBITION OF ANDROGEN-INDEPENDENT GROWTH OF PROSTATE CANCER BY SIRNA-MEDIATED ANDROGEN RECEPTOR GENE SILENCING

(PCRP PROPOSAL LOG #PC031120)

The PI: Benyi Li, MD/PhD
University of Kansas Medical Center
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Introduction

Prostate cancer is the second leading cause of cancer death among American men. Medical treatment for metastatic prostate cancer has relied heavily on androgen ablation. However, most patients treated by androgen ablation ultimately relapse to more aggressive androgen-independent cancer with no means to cure. The mechanism(s) involved in androgen-independent progression of prostate cancer is(are) not fully known, but if it were better understood, perhaps new therapies or existing ones could be used to better control prostate cancer cells. Many theories regarding the mechanism(s) for androgen-independent progression of prostate cancer have emerged, but lacking is convincing evidence to support any one of these hypotheses as the definitive mechanism. Clinically, nearly all prostate cancers retain a functional androgen receptor (AR) signaling pathway. Current evidence favors a model where activation of intracellular signal transduction pathways that stimulate the AR in the absence of ligand or in the presence of androgen antagonist. A recent report demonstrated that disruption of the androgen receptor function suppresses cellular proliferation of both androgen-dependent and -independent prostate cancer cells in an in vitro cell-based assay. Thus, it may be more clinically relevant to shift the therapeutic target from androgen to its receptor, the androgen receptor. To this end, it is extremely critical and urgent to determine if the androgen receptor is essential in androgen-independent progression of prostate cancer cells *in vivo*. The proposed studies would seek to answer the question of whether the AR signaling is essential for prostate cancer progression despite androgen deprivation. By studying what will happen if constitutively knocking down the AR expression or insight into the mechanism of androgen-independent prostate cancer will be gained. The newly developed RNAi approach gives us a powerful tool to knock down gene expression of interest per se, for example, the AR gene. The RNAi approach will determine the essential need of the AR signaling for prostate cancer cells to proliferate independent of androgen. In our preliminary studies, we found that AR siRNA against human AR gene knocked down AR protein expression in both androgen-sensitive LNCaP and androgen-insensitive PC-3/AR cells. In addition, cell growth and survival were dramatically reduced after AR siRNA transfection (as shown in *Appendix* **I**).

The objective of this proposal is to determine if AR gene silencing in prostate cancer cells via RNA interference mechanism leads to disruption of androgen-independent progression. We plan to accomplish the objective of this application by pursuing the following ***specific aims***:

(1): Generation of a recombinant AAV for long-term expression of a hairpin-structured AR siRNA *in vivo*.

(2): Determination of the essential role of the androgen receptor in androgen-independent growth of prostate cancer.

We will generate a recombinant adeno-associate virus (rAAV) for expressing the AR siRNA hairpin in a prostate cancer xenograft of animal model. Then, we will use the resultant rAAV to inject into prostate cancer xenograft established in nude mouse to determine the effect of AR gene silencing on androgen-independent growth of prostate cancer.

Report Body

In this section, we will summarize the work conducted over the entire research period outlined in the approved Statement of Work, which is listed below:

Task 1a. Generation of a recombinant AAV for the AR siRNA hairpin expression (Months 1-6)

To constitutively knockdown the endognouse *ar* gene product, a hairpin-structured construct containing an effective AR siRNA sequence (ARHP8) was generated for targeting human ar gene as shown in Fig 1A. To achieve a massive success in delivering the exogenouse DNA to cultured mammalian cells or xenografts in animal model, an Adeno-associated virus (AAV) delivery system was chosen. To simplify the procedure of AAV preparation, we changed the plan outlined in the original proposal and used a commercial available Helper-free AAV construction system to create our ARHP8-bearing AAV. This system provides a *bi*-cistronic plasmid pAAV. IRES.GFP vector for monitoring the target gene expression after inserted in the upstream of IRES-GFP cDNA sequence, which fulfills our needs as described in the proposal. So, the U6-ARHP8 expression cassette for the AR #8 siRNA hairpin was isolated from *pU6.ARHP8* plasmid vector and then inserted into the pAAV.IRES.GFP to create the pAAV-ARHP8. The resultant pAAV-ARHP8 plasmid was co-transfected with other two helping vectors (pAAV.RC and pHelper) for packaging the recombinant AAV bearing the ARHP8 (rAAV.ARHP8). A control AAV (rAAV.GFP) was made by using pAAV.IRES.GFP instead of pAAV.ARHP8. The titer of rAAVs was determined in HT1080 cells according to the manual. About 4 x 10^{12} viral particles per ml were obtained. A representative picture of AVV infection in LAPC-4 was shown in Figure 1.

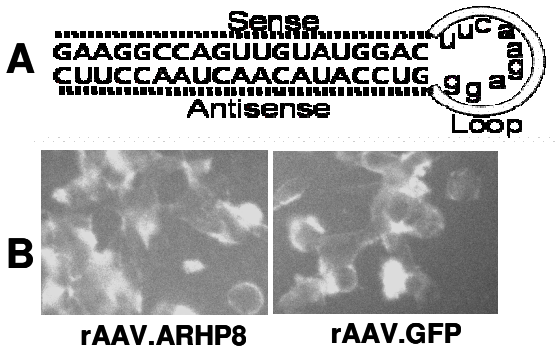


Fig. 1. AAV infection. (A) A hairpin-structured fragment used in AAV.ARHP8 preparation. (B) LAPC-4 cells were infected with AAV virus as indicated and photos were taken 3 days later under a fluorescent microscope.

Task 1b. Evaluate the efficiency of the resultant rAAV.ARHP8 for AR gene silencing (Months 7-12)

To examine if the resultant rAAV.ARHP8 is effective on knocking down AR expression, LNCaP cells were infected with the AVV at 10^5 per ml viral particles and the AR protein and mRNA levels was measured 5 days later. As shown in Fig. 2, the rAAV.ARHP8 significantly reduced AR expression both in mRNA and protein levels.

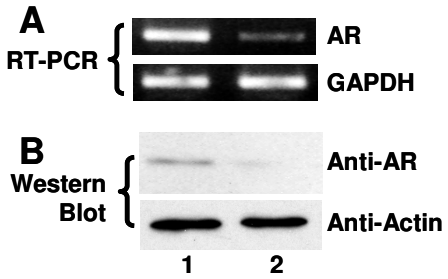


Fig. 2. ARHP8 virus induces significant knockdown of AR gene expression. After infection with rAAV.GFP (lane 1) or rAAV.ARHP8 (lane 2) for 5 days, LNCaP cells were harvested and total RNA or cellular protein were extracted for RT-PCR (A) or Western blot (B) assays as described (Ref. 1-2).

Task 2a. Pilot experiment for evaluation of rAAV.ARHP8-mediated AR gene silencing in prostate cancer xenograft of mouse model (13-19)

We first determined a proper dose for efficient distribution and knockdown AR expression in xenograft tumors, which were generated in nude mice using prostate cancer cell line PC-3AR. This cell line was described in our recent publication (Ref. 1). A total of 2.0 x 10^6 viable cells, as determined by trypan blue exclusion, was resuspended in RPMI-1640/10% fetal bovine serum (FBS) mixed with a 4:1 v/v ratio of MatriGel™ (Catalog#356237, BD Bioscience) *vs* cells and then injected subcutaneously (27-gauge needle, 1-ml disposable syringe, total volume 0.1 ml/site at 2 sites per mouse) into the rear flank of six-week old athymic male mice (Balb/c, Charles River Laboratories). Four weeks later when the tumor was palpable (around 50 mm^3 in size), 7 different doses (log-dilution, 5 x 10^3 – 5 x 10^9 viral particles in 10 µl total volume) of the recombinant rAAV.ARHP8 produced during the first year of work was injected into the tumor (multiple sites per tumor). In addition, two other animals received the control virus rAAV.GFP (2 x 10^9 viral particles in 10 µl) or 10 µl PBS as the negative controls. One week later, xenograft tumors were harvested and frozen sections were viewed for GFP expression under fluorescent microscopy. The intensity of GFP expression gradually increased along with increasing dosage of the AAV.ARHP8 particles injected. No GFP expression was observed in PBS-injected xenograft. Furthermore, Western blot results showed a consistent pattern in GFP protein levels (Fig 3, middle row in lower panel). Figures are listed in the end of this report.

In addition, we checked AR mRNA and protein levels after extracting total RNAs and proteins from xenograft specimens. As shown in Fig 3, the AR mRNA (RT-PCR) and protein levels (western blot) did not change in AAV.GFP virus-injected xenograft compared to the PBS control. However, AAV.ARHP8 virus-injected xenogarfts lost the AR expression in a virus-dosage-dependent manner. The peak effect was seen at the virus dose of 2 x 10^6 particles per 50 mm^3. Thereafter, we used this dose level for the following experiments.

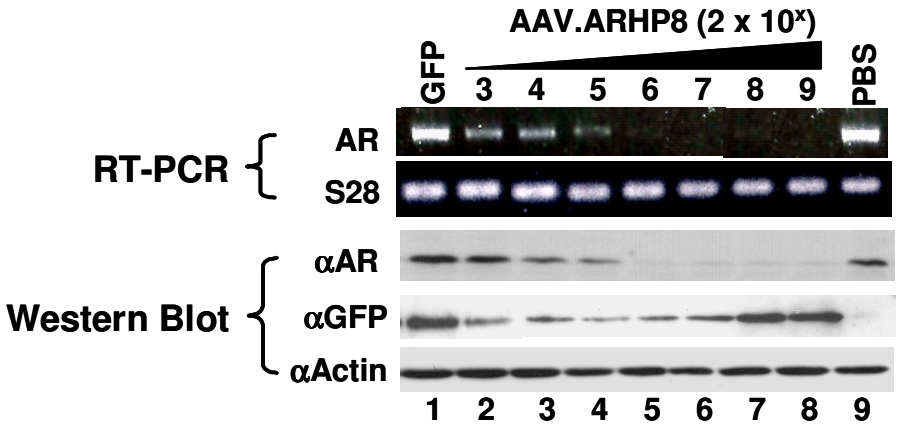


Fig 3. Determination of a proper dose of AAV.ARHP8 particle. A total of 2.0 x 10^6 PC-3AR cells per injection (s. c.) was used to established xenograft tumors in nude mice. Four weeks later when xenografts were palpable, 7 different doses (log-dilution, 2 x 10^3 – 2 x 10^9 viral particles in 10 ml, lane 2-8) of rAAV.ARHP8 was injected into the tumor (multiple sites per tumor). Empty virus rAAV.GFP (2 x 10^9 particles, lane 1) and PBS (10 ml, lane 9) were used as controls. One week later, xenograft tumors were harvested. Total RNA and protein extracts were used for detecting AR mRNA (RT-PCR assay) and AR protein (Western blot) levels as described previously (Ref 1). Meanwhile, RT-PCR for S28 gene and anti-Actin western blot served as loading control. GFP expression was also evaluated by western blot with an anti-GFP antibody (Santa Cruz Biotech).

Task 2b. Evaluate the effect of rAAV.ARHP8-mediated AR gene silencing on acquisition of the androgen-independent phenotype by prostate cancer LNCaP and LAPC-4 cells in vivo (Months 20-28)

We used the androgen-dependent prostate cancer LNCaP cell line to test if the AR is essential for tumor relapse after castration in xenograft experiments. LNCaP cells have an androgen-independent relapse phenotype after a short arresting period caused by castration (Ref. 3). Exponential growing LNCaP cells were inoculated into nude mice as described above and allowed to establish xenografts for 4-6 week. Once xenografts were palpable, animals were divided into two groups to receive bilateral castration or sham operation, respectively. One day after operation, we injected the AAV.ARHP8 or AAV.GFP virus into the xenograft tumors and monitored tumor growth for another 8 weeks. One hour before sacrifice, animals were received an intraperitoneal injection of 0.5 ml BrdU solution (10 mM, a commercial kit from Roche Diagnostics, Indianapolis, IN) for evaluating proliferation rate in the xenografts. We also measured apoptosis by means of TUNEL analysis (ApoAlert® DNA fragmentation assay kit, Cat#K2024-1, Clontech) in the tumor sections. As shown in Fig 4 (below), after castration, tumor growth was arrested for 3-4 week but resumed later on in AAV.GFP virus-injected xenografts; however, tumor growth was abolished by intratumoral injection of AAV.ARHP8 virus in both castrated and sham-operated animals.

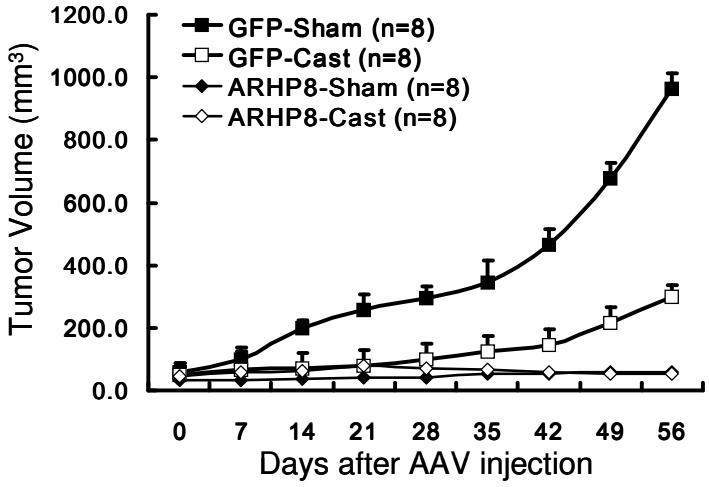


Fig 4. AAV.ARHP8 suppressed tumor growth in LNCaP xenogarfts. A total of 2 x 10^6 LNCaP cells were used to establish xenografts in nude mice and the animals were castrated or sham-operated once the xenografts were palpable. One day later, AAV.ARHP8 or AAV.GFP were injected as indicated (day 0) at a dose of 5 x 10^6 viral particles per 100 mm^3 tumor volume. Tumor growth was monitored for 8 weeks.

As shown in Fig 5 (below), a diffused GFP distribution was achieved in either AAV.ARHP8 or AAV.GFP viruses-injected xenografts. AR expression and BrdU incorporation decreased significantly in ARHP8 virus-injected but not in GFP virus-injected xenografts. A significant higher apoptotic index (TUNEL assay) was found in ARHP8 virus-treated xenografts compared to GFP virus-treated xenografts.

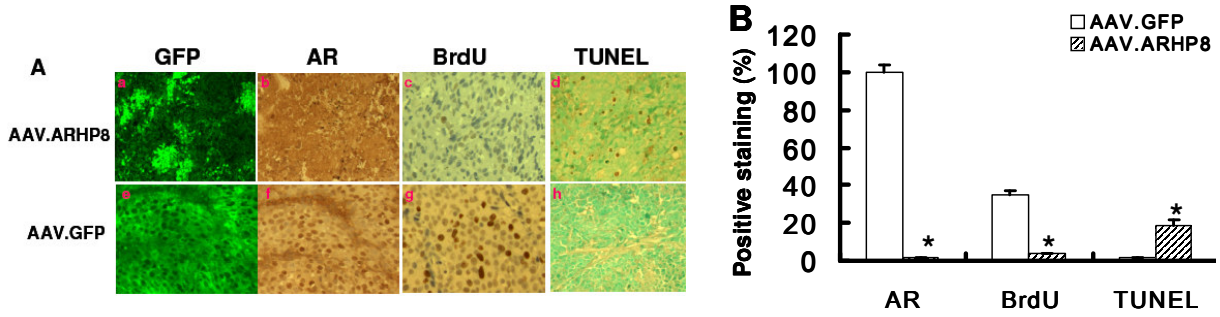


Fig 5. Analysis of GFP distribution, AR expression, BrdU incorporation and TUNEL labeling in xenograft section. A Paraffin sections were prepared from LNCaP xenograft tumors harvested from sham-operated animals. GFP distribution was assessed under fluorescent microscope; AR expression was evaluated by anti-AR (clone 441, Santa Cruz Biotech) immunostaining; BrdU incorporation was conducted with a commercial kit (Roche); TUNEL labeling was performed using ApoAlert® DNA fragmentation assay kit (Clontech). **B** Quantitative data (mean plus S.E.) were presented from 8 different xenografts.

We used the androgen-dependent prostate cancer LAPC-4 cell line to test if the AR is essential for tumor relapse after castration in xenograft experiments. Exponential growing cells were inoculated into nude mice as described above and allowed to establish xenografts for 4-6 week. Once xenografts were palpable, animals were divided into two groups to receive intra-tumoral injection of the AAV.ARHP8 or AAV.GFP virus. Tumor growth was monitored for 4 weeks. As shown in Fig 6&7, tumor growth (volume in mm^3) was abolished by intra-tumoral injection of AAV.ARHP8 virus but not AAV.GFP virus (n=8 in each group, $P < 0.01$) at 10^9 viral particles per tumor after one week of injection.

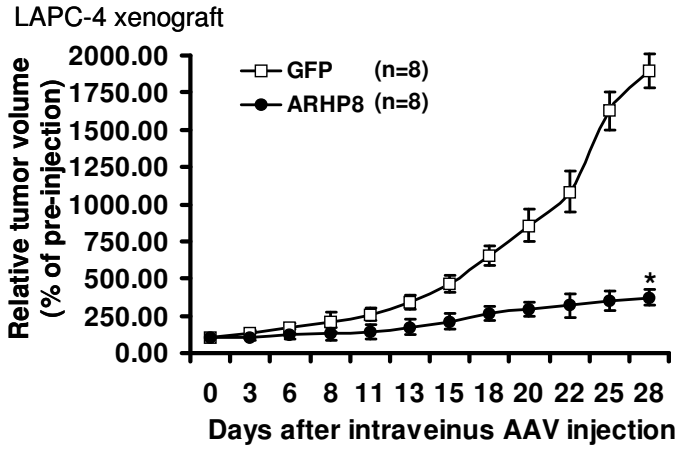


Figure 6.

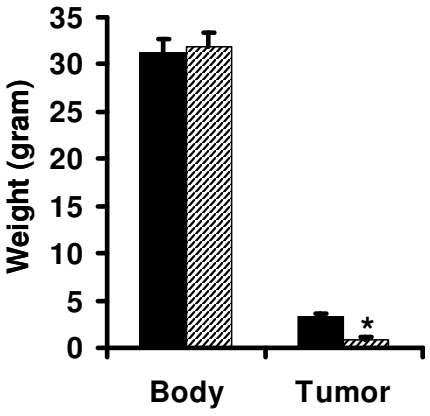


Figure 7.

Task 2c. Evaluate the effect of rAAV.ARHP8-mediated AR gene silencing on tumor growth of prostate cancer xenograft established from androgen-independent cell lines (Months 29-36)

We used the androgen-independent prostate cancer C4-2 cell line to test if the AR is essential for androgen-independent tumor growth in xenograft experiments. C4-2 cells form tumors in castrated nude mice (Ref. 4). Exponential growing C4-2 cells were inoculated into castrated nude mice as described above and allowed to establish xenografts for 4-6 week. Once xenografts were palpable, animals were divided into two groups to the AAV.ARHP8 or AAV.GFP virus injection and monitored tumor growth for another 8 weeks. BrdU incorporation assay was performed as described above. As shown in Fig 8, ARHP8 virus injection significantly abrogated tumor growth compared to GFP virus injection. Similar to that seen in LNCaP xenograft experiments as shown above, AR expression and BrdU labeling decreased dramatically in ARHP8 virus-injected xenografts compared to that in GFP virus-injected xenografts. Also, apoptotic index (TUNEL assay) was significantly higher in ARHP8 virus-injected xenografts than that in GFP virus-injected xenografts. Quantitative data was presented in Fig 9 (below).

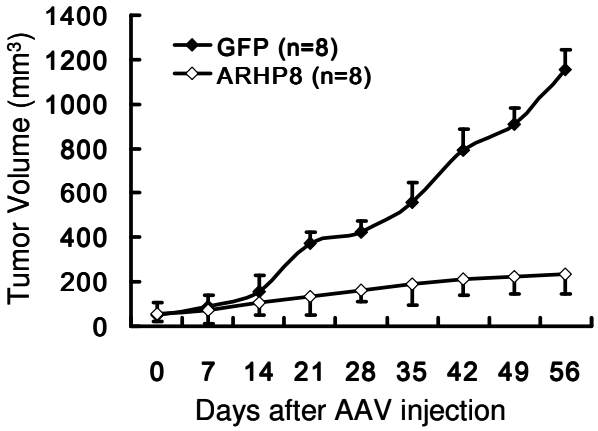


Fig 8. AAV.ARHP8 suppressed androgen-independent tumor growth in C4-2 xenogarfts. A total of 2 x 10^6 C4-2 cells were used to establish xenografts in castrated nude mice once the xenografts were palpable, AAV.ARHP8 or AAV.GFP were injected at a dose of 5 x 10^6 viral particles per 100 mm^3 tumor volume at day 0. Tumor growth was monitored for 8 weeks.

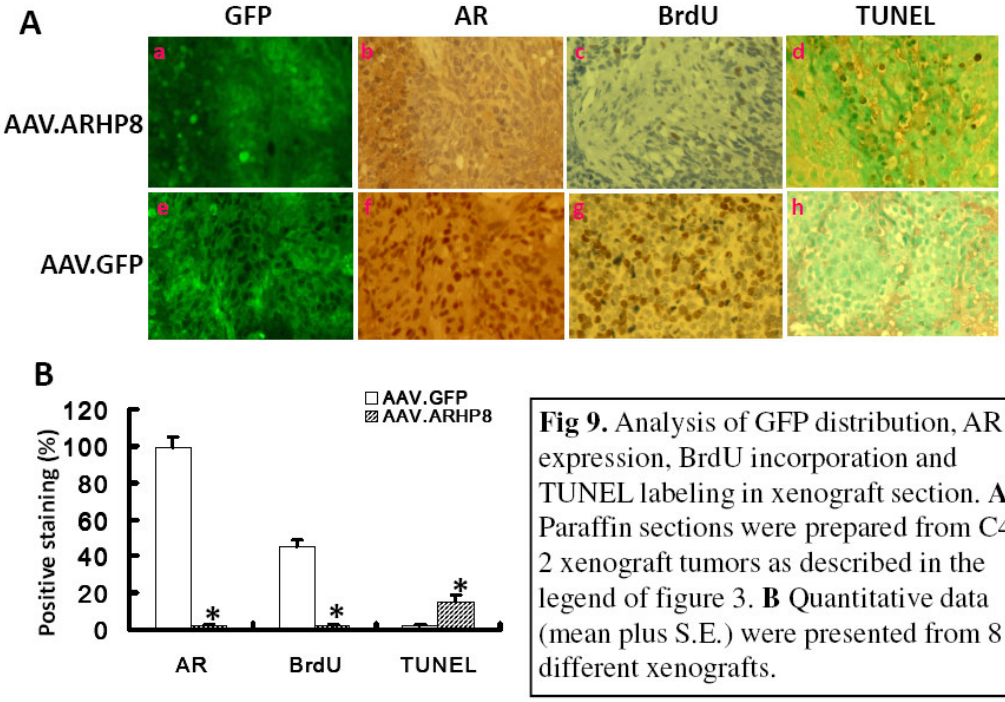


Fig 9. Analysis of GFP distribution, AR expression, BrdU incorporation and TUNEL labeling in xenograft section. **A** Paraffin sections were prepared from C4-2 xenograft tumors as described in the legend of figure 3. **B** Quantitative data (mean plus S.E.) were presented from 8 different xenografts.

Task 3. Evaluate the effect of rAAV.ARHP8-mediated AR gene silencing on tumor growth of prostate cancer xenograft established from androgen-independent cell lines (Months 37-48)

Since intra-tumoral injection of AAV-ARHP8 virus only suppressed the growth of xenograft tumors, possibly due to uneven distribution of the viruses. In order to achieve a better virus distribution and to induce a profound tumor recession, we went on to inject the viruses via tail vein for systemic delivery of the viruses. We used LAPC-4, PC-3 and CWR22Rv1 cell-derived xenografts for these experiments, as described below:

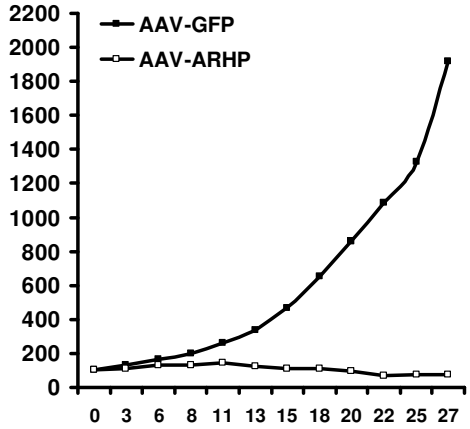


Figure 10.

First, LAPC-4 xenografts were developed in nude mice and the viruses were injected at 10^{11} viral particles per mouse per injection via tail vein. Tumor volume was monitored twice a week and relative tumor volume at each time point was calculated by comparing the volume before injection (set as 100%). As shown in Fig 10 (left), injection of AAV-ARHP virus but not AAV-GFP virus caused a significant decrease of tumor volume ($P < 0.05$). In contrast, xenograft tumors continue to growth in AAV-GFP injection.

Next, we used the CWR22Rv1 cells in the same protocol. 22RV1 is an androgen-independent prostate cancer cell line and can grow very fast even in castrated animal. Once 22R1 cells-based xenograft tumors were palpable, castrated animals were divided into two groups to the AAV-ARHP8 or AAV-GFP virus injection via tail vein (10^{11} viral particles/injection).

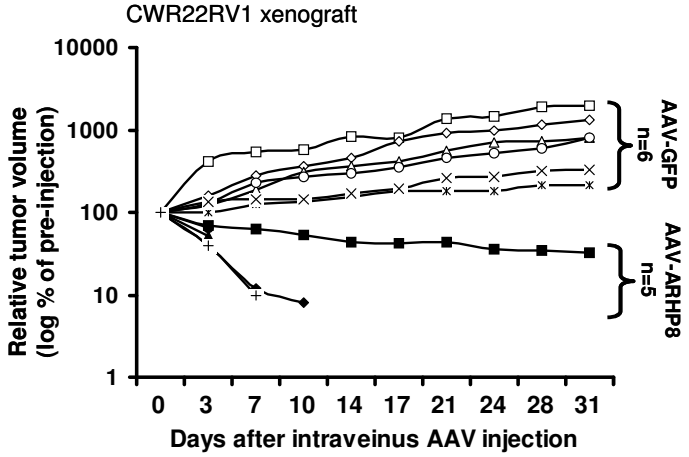
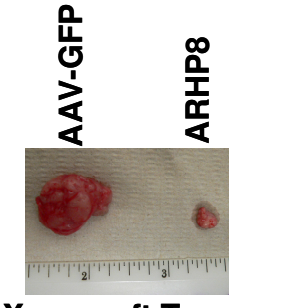


Figure 11.



Xenograft Tumors

Figure 12.

As shown in Fig 11&12, AAV-ARHP8 virus injection eradicated xenograft tumors. Three tumors disappeared within 10 days and other two tumors disappeared within 4 weeks after tail vein injection of the viruses. However, GFP virus-injected tumor showed continuous growing.

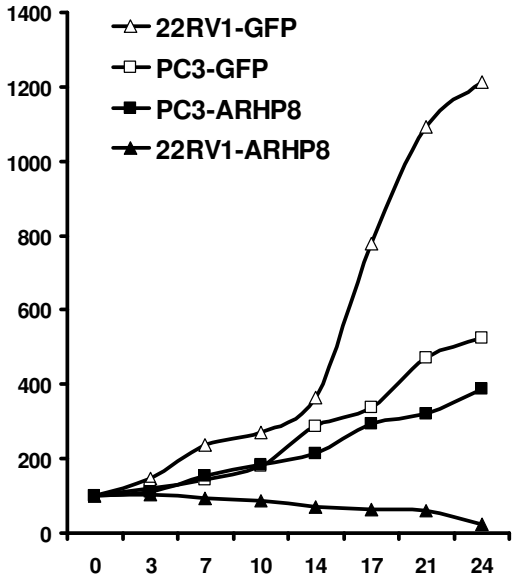


Figure 13.

Finally, we tested if virus injection affects AR-negative (PC-3) tumor growth in nude mice. Xenograft tumors were established in different sites on each animal using 22Rv1 (right side) and PC-3 (left side) cells, and then animals were injected with AAV viruses (10^{11} viral particles in 100 µl volume, n = 5 per group) via tail vein. Relative tumor volume compared to the size before injection was recorded twice a week.

As shown in Fig 13 (left), there is no significant difference in PC-3 xenograft tumors in terms of tumor volume change after virus injection between AAV-ARHP and AAV-GFP viruse injection. However, injection of AAV-ARHP viruses caused a dramatic regression of 22Rv1 xenograft tumors compared to AAV-GFP.

2. Other Achievements (Year 1-4)

a. Patent application in 2004 (*Appendix* **I**).
Benyi Li. *METHOD FOR TREATING PROSTATE CANCER USING siRNA DUPLEX FOR ANDROGEN RECEPTOR*. KUMC, December 2003.

b. Meeting abstracts based on this project:

(1) Benyi Li and Liao X*. AR silencing induced by a siRNA duplex leads to apoptosis in prostate cancer. ***AACR annual meeting 2004***, Orlando, FL.

(2) Benyi Li* and Liao X. Bcl-xL is a downstream effecter of AR-mediated survival pathway in prostate cancer. ***Keystone Symposium Feb 2005***, Monterey, CA.

(3) Benyi Li*, Xinbo Liao, J Brantley Thrasher. ANDROGEN RECEPTOR-MEDIATED REGULATION OF BCL-XL EXPRESSION: IMPLICATION IN CELLULAR SURVIVAL OF PROSTATE CANCERS. ***AUA annual meeting 2005***, San Antonio, TX. Discussed Poster - Sunday, May 22, 2005, 1:00 PM - 5:00 PM

(4) Hyewon Youn*, J. Brantley Thrasher. Gα12 is required for Androgen Receptor transactivation in prostate cancer. ***AUA annual meeting 2006***, Atlanta, GA. Discussed Poster - Monday, May 22, 2006.

(5) Benyi Li*. Serum/Glucocorticoid-induced protein kinase-1 is a downstream effect in androgen receptor-dependent survival. ***AACR Special Conference, Dec 6-9th 2006***, San Francisco, CA.

(6) Benyi Li*. Androgen Receptor siRNA eliminates prostate cancer in vivo, ***Keystone Symposium March 18-23rd 2007***, Whistler Resort, Whistler, British Columbia, Canada

(7) Jianxi Tang*, Brantley Thrasher and Benyi Li. AAV-based AR siRNA constructs eradicate prostate cancer in nude mice. ***AACR annual meeting 2008***, San Diego, CA.

(8) Jeremy Davis*, Brantley Thrasher and Benyi Li. PI3K p110β is required for androgen receptor signaling and tumor progression in prostate cancers. ***AUA annual meeting 2008***, Orlando, FL.

c. Publications (Peer-reviewed Journals)

(1) Liao X, Tang S, Thrasher JB, Griebling TL, and Benyi Li. AR siRNA-mediated androgen receptor silencing leads to apoptotic cell death in prostate cancer. ***Mol Cancer Therapeutics*** 4:505-515, 2005.

(2) Ilanchezhian Shanmugam, Guangming Cheng, Paul F. Terranova, J. Brantley Thrasher, Christie P. Thomas, and **Benyi Li**. Serum/Glucocorticoid-induced Protein Kinase-1 Facilitates Androgen Receptor-dependent Cell Survival. ***Cell Death and Differentiation***, 2007, 14(2):2085-2094.

(3) Aijing Sun, Jianxi Tang, Yan Hong, Jiawu Song, Paul F. Terranova, J. Brantley Thrasher, Stan Svojanovsky, Hong-gang Wang and **Benyi Li**. Androgen Receptor-dependent Regulation of Bcl-xL Expression: Implication in Prostate Cancer Progression. ***The Prostate***, 2008, 68(4):453-461.

(4) Qing Zhu, Jianxi Tang, Hyewon Youn, Ossama Tawfik, Paul F. Terranova, Jie Du, Patrick Raynal, J. Brantley Thrasher, and **Benyi Li**. Phosphoinositide 3-OH kinase p110β is essential for androgen receptor transactivation and tumor growth in prostate cancer. ***Oncogene***, in press, 2008.

(5) Shen FY, **Benyi Li**. [Anti-apoptotic effect of the androgen receptor in human prostate cancer]. Zhonghua Nan Ke Xue. 2007 Dec;13(12):1121-4. (review article)

d. Book Chapters.

(1) Benyi Li and J. Brantley Thrasher. Androgen Receptor and Cellular Survival in Prostate Cancer. in Recent Res. Devel. Cancer, 7(2005): ISBN: 81-7895-185-1, published by Transworld Research Network. 37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India.

(2) Benyi Li and Brantley Thrasher. "Nanotechniques in Prostate cancer Research" in <Cancer Nanotechnology>, 2006, Published by American Scientific Publishers. Valencia, California 91381-0751, USA

e. Awarded grants.

(1) **Nanoparticle-mediated delivery of AR siRNA for prostate cancer therapy**
KU Masonic Foundation Pilot Project
Principal Investigator, Total cost: $35,000, Duration: 07/2006-06/2007

(2) **Nanoparticle-mediated delivery of AR siRNA for prostate cancer intervention**
Department of Defense Prostate Cancer Research Program, Idea development Award,
Principal Investigator, Total cost: $427,416, Duration: 04/2007-03/2010

(3) **Targeted polymeric nanocarriers for combination therapy of metastatic prostate cancer**
NIH R21CA132033-01, (PI: M. Forrest, KU)
Co- Investigator, Total cost $409,041, Duration: 12/01/2007-11/30/2009

f. Personnel Trained and Supported.
Xinbo Liao, MD, Research Associate, April 2004--March 2005
Lihua Yang, PhD, postdoc fellow, November 2004--May 2005
Sandy Tang, MS, Research Assistant, March 2004--June 2006
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Jun Yang, MD, Research Fellow, Dec 2007--Feb 2008

Key Research Accomplishments

1. Generation of a recombinant adeno-associate virus (rAAV) bearing the hairpin-structured small interfering RNA against human *androgen receptor* gene (ARHP);

2. Demonstration of the efficiency of the resultant rAAV.ARHP8 in knocking down the AR expression in mRNA and protein level in prostate cancer cell LNCaP cells that express an endogenous *AR* gene.

3. Generation of another rAAV (rAAV.GFP) that lacking the ARHP8 fragment for a negative control;

4. Determination of the efficiency of other siRNA sequences (including #1, #2, #7, #34) in knocking down the AR protein level in prostate cancer LNCaP and LAPC-4 cells. The corresponding rAAV shuttle vectors are created.

5. A proper dose of AAV particle for efficient distribution and knockdown of AR gene in mouse xenograft model;

6. A total suppression of androgen-dependent tumor growth and androgen-independent transition was observed in prostate cancer LNCaP cells-derived xenografts after silencing AR gene by intra-tumoral injection of AAV.ARHP8 virus.

7. A profound suppression of androgen-independent tumor growth was achieved in prostate cancer C4-2 cells-derived xenografts after silencing AR gene by injection of AAV.ARHP8 virus.

8. Determination of two downstream effecters, Bcl-xL and SGK, and an upstream regulator PI3K p110beat in AR-mediated survival pathway.

9. A proper dose of AAV particle for efficient distribution and knockdown of AR gene in mouse xenograft model via tail vein injection;

10. A total elimination of androgen-dependent or androgen-independent xenograft tumors was observed after silencing AR gene by tail vein injection of AAV.ARHP8 virus.

11. Determination of one AR downstream effecter SGK, and an upstream regulator PI3K p110beta in AR-mediated gene expression and survival pathway.

Reportable Outcomes

1. The rAAV.ARHP8 and a control rAAV.GFP;
2. Functional AR siRNAs; including #1, #2, #5, #7, #8, #31, #34, sequence information is available upon request;
3. 5 publications in peer-reviewed journals;
4. A patent application with a title of "METHOD FOR TREATING PROSTATE CANCER USING siRNA DUPLEX FOR ANDROGEN RECEPTOR".
5. One invited review articles and two book chapters;
6. Three grants funded with the help of this project.

Conclusion

In this first-year period, we conducted the experiments according to the State of Work (month 1-12), and other related works. Based on our previous work, we generated the rAAV bearing the ARHP8 sequence and the resultant virus was demonstrated to knock down the AR in prostate cancer LNCaP cells, both at mRNA and protein levels. In addition, we tested more siRNA sequences against the AR identified by the OligoEngine software. We determined seven (out of 34 sequences) siRNAs that are potent in knocking down the AR expression. We also generated the AAV shuttle vectors bearing those sequences for future experiments.

In this second-year period, we conducted the experiments according to the State of Work (month 13-24), and other related works. Based on the outlines in the proposal, we first defined a proper dose of the rAAV bearing the ARHP8 sequence to knock down AR expression in xenografts in vivo. Then, using this dose level, we demonstrated that knocking down AR expression abolished tumor growth and blocked androgen-independent transition in LNCaP cell-derived xenografts. In addition, we observed that AR silencing resulted in a significant decrease of androgen-independent tumor growth in C4-2 cell-derived xenografts in castrated mice. These results were consistent to our recent in vitro data as described (Appendix II). We analyzed the mechanisms for AR siRNA-induced cell death and identified two anti-apoptotic proteins, Bcl-xL and SGK, as the downstream effecters of AR-mediated survival pathway.

In this third-year period, we conducted the experiments according to the State of Work (month 25-36), and other related works. Based on the outlines in the proposal, we used a dose of the rAAV bearing the ARHP8 sequence defined last year and efficiently suppressed LAPC-4 xenograft tumor growth in vivo. Then, using an alternative strategy, we demonstrated that knocking down AR expression by systemic delivery of the AAV particle eradicated androgen-independent xenograft tumors but not AR-null tumors. We continued last year's work on analyzing the mechanisms for AR siRNA-induced cell death and identified the anti-apoptotic proteins SGK1 and Bcl-xL, as the downstream effecters of AR-mediated survival pathway (Appendix III-IV).

In the fourth year period (no support extension), we continued to analyze how AR is activated after androgen ablation as well as the effect of AR silencing on tumor growth. We identified that PI3K p110beta is required for AR activation, as described in our recent article (Appendix V).

Reference

1. Xinbo Liao, Siqing Tang, J. Brantley Thrasher, Tomas Griebling, and Benyi Li. Small-interfering RNA-induced androgen receptor silencing leads to apoptotic cell death in prostate cancer. ***Mol Cancer Ther***., 2005; 4:505-515.

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Appendices

1. METHOD FOR TREATING PROSTATE CANCER USING siRNA DUPLEX FOR ANDROGEN RECEPTOR. An US patent application. Submitted on Dec 26th, 2004.
2. Liao X, Tang S, Thrasher JB, Griebling TL, and Benyi Li. AR siRNA-mediated androgen receptor silencing leads to apoptotic cell death in prostate cancer. Mol Cancer Therapeutics 4:505-515, 2005.
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4. Aijing Sun, Jianxi Tang, Yan Hong, Jiawu Song, Paul F. Terranova, J. Brantley Thrasher, Stan Svojanovsky, Hong-gang Wang and **Benyi Li**. Androgen Receptor-dependent Regulation of Bcl-xL Expression: Implication in Prostate Cancer Progression. ***The Prostate***, 2008, 68(4):453-461.
5. Qing Zhu, Jianxi Tang, Hyewon Youn, Ossama Tawfik, Paul F. Terranova, Jie Du, Patrick Raynal, J. Brantley Thrasher, and **Benyi Li**. Phosphoinositide 3-OH kinase p110β is essential for androgen receptor transactivation and tumor growth in prostate cancer. ***Oncogene***, in press, 2008.
6. Benyi Li and J. Brantley Thrasher. Androgen Receptor and Cellular Survival in Prostate Cancer. in Recent Res. Devel. Cancer, 7(2005): ISBN: 81-7895-185-1, published by Transworld Research Network. 37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India.
7. Benyi Li and Brantley Thrasher. "Nanotechniques in Prostate cancer Research" in <Cancer Nanotechnology>, 2006, Published by American Scientific Publishers. Valencia, California 91381-0751, USA

SUPPORTING DOCUMENT FOR PROGRESS STATEMENT (B)

UNITED STATES PATENT APPLICATION FOR:

METHOD FOR TREATING PROSTATE CANCER USING siRNA DUPLEX FOR ANDROGEN RECEPTOR

INVENTOR:

Benyi Li

Attorney Docket No.: 506275-0022

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METHOD FOR TREATING PROSTATE CANCER

USING siRNA DUPLEX FOR ANDROGEN RECEPTOR

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application claims the benefit of U.S. Provisional Application No. 60/531,881 filed December 22, 2003, which is hereby incorporated by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[002] Not Applicable.

BACKGROUND OF THE INVENTION

[003] 1. FIELD OF THE INVENTION

[004] The present invention is directed to a method of treating prostate cancer using interfering RNA duplexes to mediate gene silencing.

[005] 2. DESCRIPTION OF RELATED ART

[006] Prostate cancer is a significant risk for men in the United States. Sixty years ago, it was found that androgens were required for prostate epithelial cells to proliferate, differentiate, and survive. In addition, apoptotic cell death has been found in the prostate after androgen withdrawal.

[007] Because of this insight, androgen ablation has been widely accepted as a major medical treatment for metastatic prostate cancer. However, most patients treated by androgen ablation ultimately relapse to more aggressive incurable androgen-refractory prostate cancer.

[008] Anti-androgen withdrawal syndrome is another concern for androgen antagonist therapy. The etiology of androgen-independent relapse may have various molecular causes, but in each scenario, the androgen receptor ("AR") is expressed and its function is maintained, suggesting that androgen-independent AR signaling is involved. In a transgenic mouse model, AR overexpression in prostate epithelium resulted in marked increases in epithelial proliferation and focal areas of intraepithelial neoplasia in the ventral prostate and dorsolateral prostate. Recently, the critical role of the AR for cellular proliferation *in vitro* or tumor growth *in vivo* of prostate cancer has been demonstrated by several different approaches, including disruption of AR function by anti-AR antibody and the reduction of AR expression by AR specific ribozyme or antisense oligonucleotides (Zegarra-Moro 2002, Eder 2000, Eder 2002). However, the role of the AR in cellular survival remains unknown in prostate cancer.

[009] Apoptosis, or programmed cell death, is a well-conserved process whose basic tenets remain common to all metazoans (Hengartner 2000, Danial 2004). Intracellular organelles, like mitochondria, are key participants in apoptosis. The main aspects of mitochondrial involvement in apoptotic process include two critical events: (1) the release of mitochondrial proteins, including cytochrome c and (2) the onset of multiple parameters of mitochondrial dysfunction, such as loss of membrane potential. The Bcl-2 family proteins are critical regulators that directly control the mitochondria function and consist of both pro-apoptotic and anti-apoptotic members (Boise 1993). Bax, Bak, and Bok are pro-apoptotic members, as are the BH3-domain only members such as Bad, Bik, and Bid. Anti-apoptotic members include Bcl-2 and Bcl-x_L, Bcl-w, and Mcl-1. It is believed that the relative levels of pro-apoptotic and anti-apoptotic members are the key determinants in the regulation of cell death and survival.

[010] The *bcl-x* gene encodes multiple spliced mRNAs, of which Bcl-x_L is the major transcript (Boise 1993, Gonzalez-Garcia 1994). Like Bcl-2, Bcl-x_L protects cells from apoptosis by regulating mitochondria membrane potential and volume, and subsequently prevents the release of cytochrome c

and other mitochondrial factors from the intermembrane space into cytosol. In addition, Bcl-x_L may prevent apoptosis via a cytochrome c-independent pathway (Li, F. 1997). Although Bcl-x_L protein can be regulated post-transcriptionally, it is mainly controlled at the gene expression level (Grad 2000). Bcl-x_L protein is detected in the epithelial cells of normal prostate gland and prostate cancers and the expression level of Bcl-x_L protein correlated with higher grade and stage of the disease, indicating an important role of Bcl-x_L in prostate cancer progression (Krajewska 1996).

[011] RNA interference ("RNAi") is a recently discovered mechanism of post-transcriptional gene silencing in which double-stranded RNA corresponding to a gene (or coding region) of interest is introduced into an organism, resulting in degradation of the corresponding mRNA. The phenomenon was originally discovered in *Caenorhabditis elegans* by Fire and Mello.

[012] Unlike antisense technology, the RNAi phenomenon persists for multiple cell divisions before gene expression is regained. The process occurs in at least two steps: an endogenous ribonuclease cleaves the longer dsRNA into shorter, 21- 22- or 23-nucleotide-long RNAs, termed "small interfering RNAs" or siRNAs (Hannon 2002). The siRNA segments then mediate the degradation of the target mRNA. RNAi has been used for gene function determination in a manner similar to but more efficient than antisense oligonucleotides. By making targeted knockouts at the RNA level by RNAi, rather than at the DNA level using conventional gene knockout technology, a vast number of genes can be assayed quickly and efficiently. RNAi is therefore an extremely powerful, simple method for assaying gene function.

[013] RNA interference has been shown to be effective in cultured mammalian cells. In most methods described to date, RNA interference is carried out by introducing double-stranded RNA into cells by microinjection or by soaking cultured cells in a solution of double-stranded RNA, as well as transfecting the cells with a plasmid carrying a hairpin-structured siRNA expressing cassette under the control of suitable promoters, such as the U6, H1 or cytomegalovirus ("CMV") promoter (Sui 2002, Paddison 2002, Yu 2002, Zia 2002, Brummelkamp 2002, Harborth 2001, Elbashir 2001, Miyagishi 2002, Lee 2001, Paul 2002). The gene-specific inhibition of gene expression by double-stranded ribonucleic acid is generally described in Fire et al., U.S. Patent No. 6,506,559, which is incorporated by reference. Exemplary use of siRNA technology is further described in McSwiggen, Published U.S. Patent Application No. 2003/01090635 and Reich et al., Published U.S. Patent Application No. 20040248174, which are incorporated by reference.

BRIEF SUMMARY OF THE INVENTION

[014] An object of the present invention is to develop a gene therapeutic strategy for treating prostate cancer.

[015] Another object of the present invention is to provide a method for treating cancer which results in apoptotic cell death.

[016] Another object of the present invention is to use the RNA interference technique to achieve a profound *AR* gene silencing in prostate cancer cells that subsequently leads to apoptosis as evidenced by increased caspase-3 activation.

[017] Yet another object of the present invention is to use the RNA interference technique to achieve a profound *AR* gene silencing in prostate cancer cells that subsequently leads to apoptosis as evidenced by increased poly (ADP)-ribose polymer (PARP) cleavage.

[018] Yet another object of the present invention is to use the RNA interference technique to achieve a profound *AR* gene silencing in prostate cancer cells that subsequently leads to apoptosis as evidenced by a reduction of the anti-apoptotic protein Bcl-xL.

[019] Additional aspects of the invention, together with the advantages and novel features appurtenant thereto, will be set forth in part in the description which follows, and in part will become apparent to those skilled in the art upon examination of the following, or may be learned from the practice of the invention. The objects and advantages of the invention may be realized and attained by means of the instrumentalities and combinations particularly pointed out in the appended claims.

[Cover Caption]

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Small-interfering RNA–induced androgen receptor silencing leads to apoptotic cell death in prostate cancer

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Abstract

Prostate cancer is the second leading cause of cancer death in the United States and, thus far, there has been no effective therapy for the treatment of hormone-refractory disease. Recently, the androgen receptor (AR) has been shown to play a critical role in the development and progression of the disease. In this report, we showed that knocking down the AR protein level by a small interfering RNA (siRNA) approach resulted in a significant apoptotic cell death as evidenced by an increased annexin V binding, reduced mitochondrial potential, caspase-3/6 activation, and DFF45 and poly(ADP-ribose) polymerase cleavage. The apoptotic response was specifically observed in those siRNA-transfected cells that harbor a native *AR* gene. No cell death was found in the AR-null prostate cancer cell PC-3 or its subline that has been reconstituted with an exogenous *AR* gene, as well as two breast cancer cell lines that are AR positive. Moreover, in parallel with the siRNA-induced AR silencing, the anti-apoptotic protein Bcl-xL was significantly reduced, which might account for the apoptotic cell death because ectopic enforced expression of Bcl-xL protein partially inhibited apoptosis after AR silencing. Taken together, our data showed that knocking down the AR protein level in prostate cancer cells leads to apoptosis by disrupting the Bcl-xL–mediated survival signal downstream of AR-dependent survival pathway. [Mol Cancer Ther 2005; 4(4):505–15]

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Introduction

Prostate cancer is a significant risk for men in the United States (1). Sixty years ago, it was found that androgens were required for prostate epithelial cells to proliferate, differentiate, and survive; apoptotic cell death has been found in the prostate after androgen withdrawal (2, 3). Because of this insight, androgen ablation has been widely accepted as a major medical treatment for metastatic prostate cancer. However, most patients treated by androgen ablation ultimately relapse to more aggressive incurable hormone refractory prostate cancer (4). Moreover, antiandrogen withdrawal syndrome is another concern for androgen antagonist therapy (5). The etiology of hormone-refractory relapse may have various molecular causes, but in each scenario the androgen receptor (AR) is expressed and its function is maintained (6–11), suggesting that androgen-independent AR signaling is involved. In a transgenic mouse model, AR overexpression in prostate epithelium resulted in marked increases in epithelial proliferation and focal areas of intraepithelial neoplasia in the ventral prostate and dorsolateral prostate (12). Recently, the critical role of the AR for cellular proliferation *in vitro* or tumor growth *in vivo* of prostate cancer has been shown by different approaches, including disruption of AR function by anti-AR antibody, inhibition of AR expression by AR-specific ribozyme or antisense oligonucleotides, as well as knocking down AR expression by the RNA interference (RNAi) approach (8, 13–15). However, the mechanisms of AR-dependent cellular survival remain unclear in prostate cancer progression although some survival mechanisms involved in hormone-resistant progression of prostate cancer have been proposed (16–20).

Apoptosis, or programmed cell death, is a well-conserved process whose basic tenets remain common to all metazoans (21, 22). Intracellular organelles, like mitochondria, are key participants in apoptosis. The main aspects of mitochondrial involvement in apoptotic process include two critical events, the onset of multiple parameters of mitochondrial dysfunction, such as loss of membrane potential and the release of mitochondrial proteins including cytochrome *c*. The Bcl-2 family proteins are critical regulators that directly control the mitochondrial function and consist of both proapoptotic and antiapoptotic members (23). Bax, Bak, and Bok are proapoptotic members, as are the BH3 domain only members, such as Bad, Bik, and Bid. Antiapoptotic members include Bcl-2 and Bcl-xL, Bcl-w, Mcl-1, etc. It is believed that the relative levels of proapoptotic and antiapoptotic members are the key determinants in the regulation of cell death and survival.

The *bcl-x* gene encodes multiple spliced mRNAs, of which Bcl-xL is the major transcript (23, 24). Like Bcl-2, Bcl-xL protects cells from apoptosis by regulating mitochondrial membrane potential and volume, and subsequently prevents the release of cytochrome *c* and other mitochondrial factors from the intermembrane space into cytoplasm. In addition, Bcl-xL may prevent apoptosis via a cytochrome *c*–independent pathway (25). Although Bcl-xL protein can be regulated posttranscriptionally, it is mainly controlled at the gene expression level (26, 27). Bcl-xL protein is detected in the epithelial cells of normal prostate gland and prostate cancers in an earlier report (28). The expression level of Bcl-xL protein correlated with higher grade and stage of the disease, indicating an important role of Bcl-xL in prostate cancer progression.

RNAi is a recently discovered mechanism of posttranscriptional gene silencing in which double-stranded RNA corresponding to a gene (or coding region) of interest is introduced into an organism, resulting in degradation of the corresponding mRNA (29, 30). Unlike antisense technology, the RNAi phenomenon persists for multiple cell divisions before gene expression is regained, and is more efficient than antisense oligonucleotides. RNAi is, therefore, an extremely powerful, simple method for assaying gene function (31).

In an effort to dissect the mechanism of AR-dependent survival and to develop novel therapeutic strategies for prostate cancer, we knocked down the AR protein expression in prostate cancer cells that harbor the *AR* gene using the RNAi technique. Surprisingly, in addition to cell arrest, we found a significant apoptotic cell death when AR expression was knocked down by a small interfering RNA (siRNA) duplex. Most interestingly, the antiapoptotic protein Bcl-xL was also decreased in parallel with AR silencing, and overexpression of exogenous Bcl-xL controlled by a cytomegalovirus promoter partially rescued the cells from AR siRNA-induced apoptosis.

Materials and Methods

Cell Lines and Reagents

The human prostate cancer LNCaP, LAPC-4, PC-3, C4-2 and CWR22Rv1 cells, and HEK293 cells were described previously (32–34). The cell line information is briefly summarized in Table 1. Prostate epithelial cell RWPE-1 and breast cancer cell lines MCF-7 and T47D were obtained from American Type Culture Collection (Manassas, VA). The hormone-refractory prostate cancer cell LNCaP-Rf was a kind gift provided by Dr. Donald Tindall (Department of Biochemistry, Mayo Clinic, Rochester, MN; ref. 13). PC-3/AR subline was established by stably transfecting the AR-null PC-3 cells with a vector bearing the human *AR* gene obtained from Dr. Fahri Saatcioglu (Department of Biology, University of Oslo, Oslo, Norway). PC-3/Neo subline was established when an empty vector was used. The stable clones were selected in G418 and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). LNCaP/Bcl-xL subline was established by stably transfecting the LNCaP cells with a vector bearing the human *bcl-xl* cDNA sequence with a HA-tag obtained from Dr. Hong-gang Wang (Moffitt Cancer Center, University of South Florida, Tampa, FL) and LNCaP/puromycin subline was established when an empty vector was used. The stable clones were selected in a puromycin-containing culture medium. Antibodies against human AR, actin, and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against caspases, cytochrome *c*, Bcl-2 family members, XIAP, DFF45, and poly(ADP-ribose) polymerase were obtained from Cell Signaling (Beverly, MA). JC-1 fluorescent dye was obtained from Molecular Probes (Eugene, OR). Charcoal-stripped FBS was obtained from Atlanta Biologicals (Norcross, GA). Other reagents were supplied by Sigma (St. Louis, MO).

siRNA Synthesis, Labeling, and Transfection

Sequence information regarding the human *AR* gene (Genbank accession no. NM_000044) was extracted from the National Center for Biotechnology Information Entrez nucleotide database. Up to 34 mRNA segments were identified using the OligoEngine software (OligoEngine, Inc., Seattle, WA), which fulfill the requirements for potentially triggering RNAi according to the literature (31). The *AR* gene specificity was confirmed by searching the National Center for Biotechnology Information BlastN database. The siRNAs were prepared by a transcription-based method using the *Silencer* siRNA construction kit (Ambion, Austin, TX) according to the manufacturer's instructions. The 29-mer sense and antisense DNA oligonucleotide templates (21 nucleotides specific to the targets and 8 nucleotides specific to T7 promoter primer sequence 5′-CCTGTCTC-3′) were synthesized by IDT (Coralville, IA). The quality of the synthesized siRNA was estimated by agarose gel analysis and found to be very clean. RNAs were quantified by using RiboGreen fluorescence (Molecular Probes). A Silencer siRNA labeling kit using a fluorescent Cy3 dye (Ambion) was used for labeling the siRNA duplexes according to the manufacturer's instructions. The purified siRNA duplexes were transfected into cells with the Oligofectamine reagent (Invitrogen, Co., Carlsbad, CA) in a medium supplied with 2% charcoal-stripped FBS. The media were changed every 3 days. A scrambled negative siRNA duplex (Ambion) was used as control. A pooled chemically synthesized AR siRNA mixture was purchased from Upstate (Charlottesville, VA).

Western Blotting and Immunofluorescence Staining

For Western blot, cells were washed in PBS and lysed in a radioimmunoprecipitation assay buffer supplied with protease inhibitors (CytoSignal, Irvine, CA). Western blot analysis was done as described previously (32–35) to assess the protein expression level of target molecules. Blots were developed with a SuperSignal West Dura substrate kit (Pierce Biotech, Rockford, IL). Immunofluorescent staining was done as previously described (34, 35). The picture was taken under a fluorescence microscope (Nikon Inc., Melville, NY) set at ×200 magnification.

Table 1. Summary of cell lines used in this study

Cell line	Origin and modification	AR status	Hormone response	Reference
LNCaP	Human prostate cancer	Mutant	Yes	55
LAPC-4	Human prostate cancer	Wild type	Yes	56
PC-3	Human prostate cancer	Null	No	57
C4-2	LNCaP coengrafted with bone marrow cells	Mutant	No	58
CWR22Rv1	Human prostate cancer	Mutant	No	42
RWPE-1	Human prostate epithelium transformed by HPV-18	Wild type	Yes	41
HEK293	Human embryo kidney transformed by adenovirus 5	Null	No	59
MCF-7	Human breast cancer	Positive	Yes	60, 61
T47D	Human breast cancer	Positive	No	62

Cytotoxicity Assays and Flow Cytometry

Typically, cell viability was assessed with a trypan blue exclusion assay as described in our previous publication (33). Apoptotic cell death was determined using an annexin V-FITC Apoptosis Detection kit (BD PharMingen, San Diego, CA) according to the manufacturer's manual. Briefly, cells were harvested and washed with ice-cold PBS and then suspended in annexin V binding buffer. Then, cells were stained for 15 minutes at room temperature in the dark and analyzed on a FACSCalibur flow cytometer using CELLQuest software. For clonogenic survival assay, 10^3 cells were seeded in a 35 mm dish and transfected with the siRNAs as indicated in the figure legend. The media were changed every 3 days and the cultures were observed daily for colony formation. On day 7, the cultures were washed with PBS, fixed, and stained as previously described (36). The colonies were counted under an inverted microscope.

mRNA Expression Analysis and Reverse Transcription-PCR

Total RNA was prepared using Trizol reagent (Invitrogen). To assess mRNA expression, a semiquantitative reverse transcription-PCR (RT-PCR) method was used as described previously (35). RT-PCR was done using a RETROscript kit (Ambion) per manufacturer's instructions. The primers and PCR conditions were described as follows: for human *AR* gene (forward 5′-cctggcttccgcaacttacac-3′; backward 5′-ggacttgtgcatgcggtactca-3′; adapted from ref. 6); human *PSA* gene (forward 5′-gatgactccagccacgacct-3′; backward 5′-cacagacaccccatcctatc-3′; ref. 37); and human *bcl-xl* gene (forward 5′-catggcagcagtaaagcaag-3′; backward 5′-gcattgttcccatagagttcc-3′; ref. 38). 28S ribozyme RNA (forward 5′-gttcacccactaatagggaac gtg-3′; backward 5′-gattctgacttagaggcgttcagt-3′) was used as an internal control. The primers were synthesized by IDT. The amplification profile was as follows: 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute running in a total of in 25 cycles. After 25 amplification cycles, the expected PCR products were size fractionated onto a 2% agarose gel and stained with ethidium bromide.

Mitochondrial Membrane Potential and Caspase Activity

The siRNA-transfected cells were incubated in the presence of JC-1, which was added to the culture medium at a final concentration of 0.3 μg/mL for 15 minutes at 37°C. Thereafter, the cells were analyzed under a fluorescent microscope. The caspase activity was measured using an Apo-ONE Homogeneous Caspase-3/7 Assay kit obtained from Promega (Madison, WI) per the manufacturer's manual. Briefly, the cells were washed in ice-cold PBS and then suspended in the assay buffer containing the substrate rhodamine 110 (Z-DEVD-R110) provided by the supplier. The amount of fluorescent product generated is measured at 480/520 nM (wavelength) using a Fluoscan fluorescent reader as described previously (32, 34).

Statistical Analysis

All experiments were repeated twice or thrice. Western blot results are presented from a representative experiment. The mean and SD from two experiments for cell viability are shown. The number of viable/dying cells or cell colonies in the control group or the initial time point was assigned a relative value of 100%. The significant differences between groups were analyzed using the SPSS computer software (SPSS, Inc., Chicago, IL).

Results

Knocking Down AR Expression via RNA Interference Approach in Prostate Cancer Cells

Because the AR has been shown to play a critical role in hormone-refractory progression of prostate cancer (6–17), targeting the *AR* gene by reducing its translation or blocking its function via antisense approach has emerged as a novel strategy for prostate cancer therapy (13–15). Recently, RNA interference has been shown to be a better strategy in blocking gene expression in cultured cells or animal model (29–31). To explore the feasibility of the RNAi technique in knocking down AR expression in prostate

cancer cells that harbor the *AR* gene, we designed and synthesized a panel of siRNAs against human *AR* gene. Two relatively potent siRNAs (AR siRNA 8, 5′-AAGAAG-GCCAGUUGUAUGGAC-3′; AR siRNA 31, 5′-AAGACG-CUUCUACCAGCUCAC-3′) were identified in knocking down AR expression in the initial experiments when compared with others. The AR knockdown effect was further confirmed by checking the mRNA level followed by Western blot. A well-known androgen target prostate-specific antigen was also down-regulated as determined by a RT-PCR assay. This knocking down effect was achieved as a sequence-specific event because a negative control siRNA with a scrambled sequence had no effect on AR protein or prostate-specific antigen mRNA level (Fig. 1A). Both AR siRNAs 8 and 31 significantly knocked down AR expression at a final concentration of 1.0 to 10 nmol/L in culture media after 4 days in LNCaP cells that harbor an endogenous mutant *AR* gene, as well as in PC-3/AR cells that were reconstituted with an exogenous wild-type *AR* gene (Fig. 1B). Moreover, the knocking down effect of the AR protein was further verified using an immunofluorescent staining approach where LAPC4 cells, which harbor an endogenous wild-type *AR* gene, were used (Fig. 1C). These results show that the RNAi machinery is functional in prostate cancer cells, which is consistent with two recent reports (8, 39), and can be activated by a siRNA duplex.

siRNA-Mediated AR Silencing Leads to Dramatic Cell Death

It is shown that the AR is a key factor for cell proliferation *in vitro* (13, 14) or tumor growth *in vivo* (15) in prostate cancer. Consistent with two recent reports showing a reduced cell proliferation after AR protein was knocked down via the RNAi approach (8, 39), we also found that cell growth was largely reduced after transfection of LAPC-4 cells with either AR siRNA 8 or a pooled AR siRNA mixture (Fig. 2A). However, the difference was that a massive cell death was observed if the cells were monitored for >4 days after siRNA transfection. To test if the cell death response is due to siRNA-mediated AR silencing, we did a time course experiment in LNCaP (hormone-sensitive) and C4-2 (hormone-refractory) cells. The cells were transfected with AR siRNA 8 or a scrambled negative siRNA in 2% charcoal-stripped FBS. The relative survival rate of the cells was determined every 2 days using a trypan blue exclusion assay. Transfection of the cells with the AR siRNA duplexes resulted in a significant cell death in which LNCaP cells (Fig. 2B) showed a quicker response compared with C4-2 cells (Fig. 2C). In contrast, the negative control siRNA did not cause cell death. These data suggest that the AR siRNA induces cell death regardless of hormone sensitivity, although C4-2 cells showed a delayed response compared with LNCaP cells.

Next, we asked if the AR siRNA–induced cell death was simply due to a cellular nonspecific response to the double-stranded siRNA (i.e., IFN response; ref. 40) or those degraded AR mRNA produced by the RNAi machinery. The experiments were conducted using PC-3/AR, PC-3/Neo (empty vector control subline), and LNCaP-Rf (hormone-refractory, ref. 13) cell lines. As shown in Fig. 2D, either AR siRNA 8 or 31 significantly reduced the survival rate for >95% in LNCaP-Rf cells compared with the control siRNA. In contrast, the cell survival rate

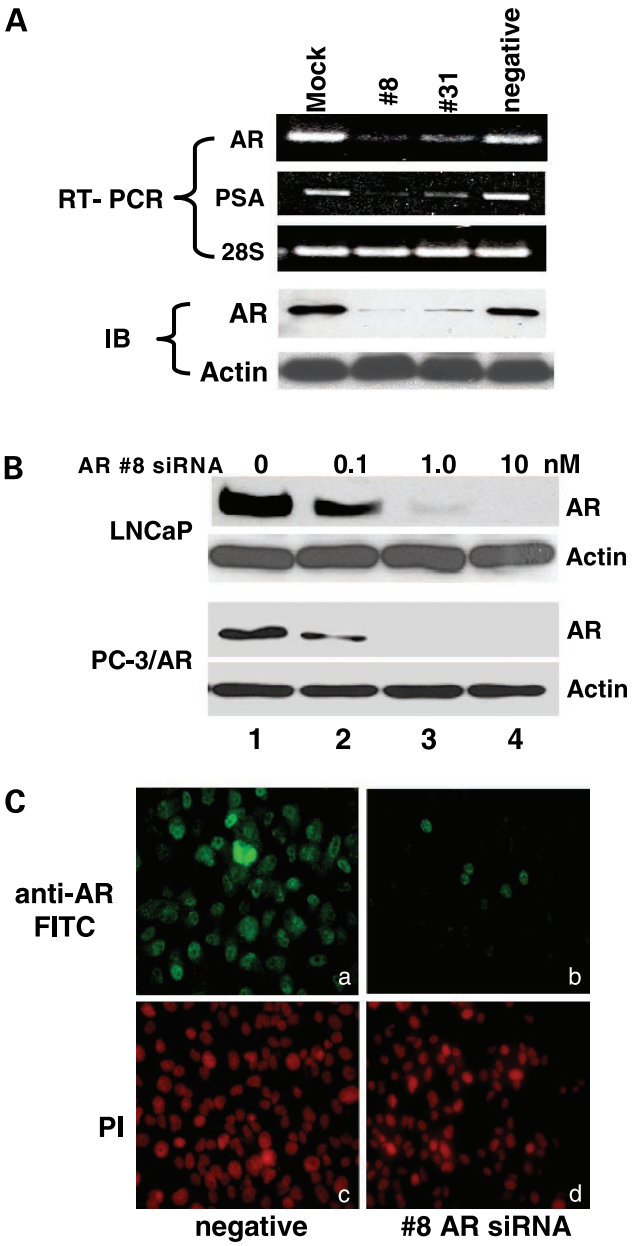

Figure 1. siRNA-mediated *AR* gene silencing in prostate cancer cells. **A,** following transfection with the siRNA duplexes (final concentration at 10 nmol/L in the medium) as indicated, cells were harvested at 48 h (*top*) or 72 h (*bottom*) later. The mRNA levels of target genes as indicated were determined by RT-PCR assay (*top*) and the AR protein was determined by Western blot (*bottom*). Actin blot served as loading control. The siRNA was omitted in the mock control. **B,** cells were transfected with different concentrations of AR siRNA 8 and then harvested 72 h later. Western blot was done as above. **C,** LAPC-4 cells were transfected with the siRNA duplexes (10 nmol/L in the medium) as indicated for 72 h and then subjected to immunofluorescent staining as described in the text.

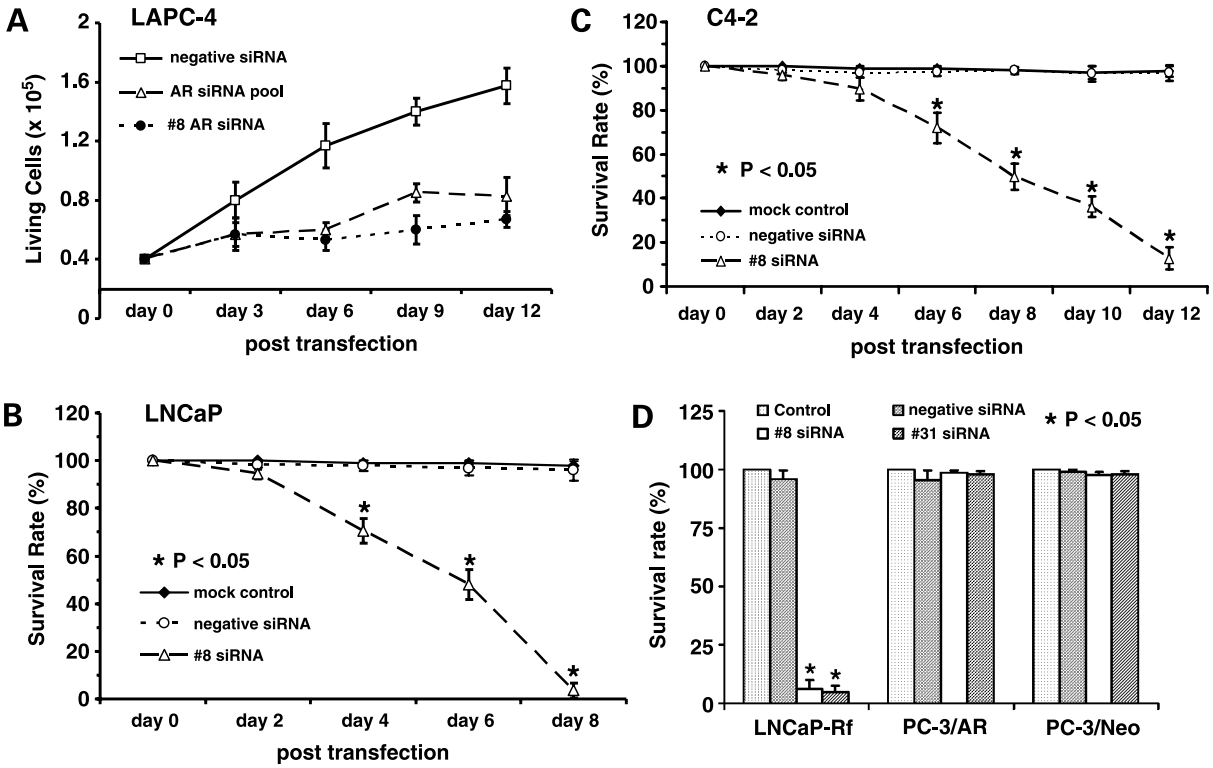

Figure 2. AR siRNA induces cell arrest and death. Cells seeded in six-well plates were transfected with the siRNAs (10 nmol/L in the medium) as indicated. **A,** the total number of living (white or unstained) cells from each time point was counted by trypan blue exclusion assay. **B** and **C,** the survival rate (white cells versus white plus blue cells) was determined in each time point by trypan blue exclusion assay, and then the relative survival rate was calculated by normalizing the data from late time points against the initial time point group that was set as 100%. **D,** cells were seeded in 35 mm dishes at a density of 10^3 cells per dish overnight and then transfected with the siRNA duplexes (10 nmol/L in the medium) as indicated. The clonogenic survival fraction of the cells was determined on day 7 posttransfection. Colonies were fixed, stained, and counted. The survival rate in control group was designated as 100%. Data are from three different experiments.

was not affected in either PC-3/AR or PC-3/Neo cells after the siRNA transfection. These data suggest that the AR siRNA–induced cell death in the native AR-harboring cells is not a nonspecific cellular response to the double-stranded siRNA or siRNA-mediated AR mRNA degradation but due to a disruption of the survival machinery that depends on the AR. In the AR-null cells, like PC-3/Neo or PC-3/AR cells where an exogenous *AR* gene is expressed, the survival machinery of the cells might not depend on the AR.

AR siRNA–Induced Cell Death Occurs Specifically in Prostate-Derived Cells

In addition to those commonly used prostate cancer cells as mentioned above, we also tested the cell death response to the AR siRNA in two more prostate epithelial cell lines (RWPE-1 and CWR22Rv1) and breast cancer cell lines (MCF-7 and T47D) to verify the specificity of AR siRNA–induced cell death. The RWPE 1 is a nontumorigenic prostate epithelial cell line (41), whereas the CWR22Rv1 is a hormone-refractory prostate cancer cell derived from CWR22 xenograft (42). Although the CWR22Rv1 cells, like C4-2 cells, showed a delayed response to AR siRNA–induced cell death, the nontumorigenic RWPE-1 cell line showed a rapid death response even faster than LAPC-4 (Fig. 3A) and LNCaP cells (Fig. 2B). However, the two breast cell lines did not show any cell death response to the AR siRNA although they are also harboring an endogenous AR (data not shown). A selected data for AR siRNA–induced AR protein knockdown in CWR22Rv1 and LAPC-4 cells was shown in Fig. 3B.

To visualize the specificity of the AR siRNA–induced cell death, we labeled AR siRNA 31 with a fluorescent dye (Cy3) and then transfected into LNCaP cells. Cells were maintained in 2% charcoal-stripped FBS and cell death was monitored daily under a fluorescent microscope. As shown in Fig. 4, the Cy3-labeled siRNA was seen in a large population of the cells, indicating a successful transfection. Most interestingly, only the dying cells (round and detached from the plastic) showed a positive Cy3 labeling (Fig. 4, *black arrow*); however, living cells (spreading and attached cells) showed no Cy3 labeling (Fig. 4, *white arrow*). These data show the specific effect of the AR siRNA–induced cell death only on the transfected cells.

Mitochondrial Apoptotic Mechanism Is Involved in AR siRNA–Induced Cell Death

It has been shown that androgen ablation or antiandrogens induces apoptotic cell death in prostate epithelium and

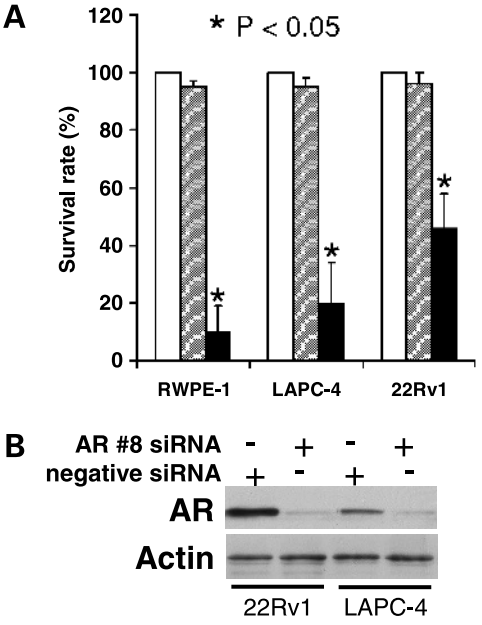

Figure 3. AR siRNA specifically induces cell death in other prostate-derived cells. **A,** three prostate cell lines (RWPE-1, LAPC-4, and CWR22Rv1) were transfected with AR siRNA 8 at 10 nmol/L in the culture medium supplied with 2% charcoal-stripped FBS, and cell survival rate was determined 7 days later by trypan blue exclusion assay. **B,** cells harvested from the experiments described in (**A**) were lysed for Western blot analysis to determine the protein levels of the AR. Actin blot served as loading control.

prostate cancer cells (3). To determine if AR siRNA–induced cell death is an apoptotic response, we first detected the change of the membrane phospholipid phosphatidylserine, which is translocated from the inner to the outer leaflet of the plasma membrane during the earlier phase of apoptosis (43). As shown in Fig. 5A, transfection of the cells with the AR siRNAs induced significant phosphatidylserine translocation, whereas the control siRNA had no effect.

Because loss of mitochondrial transmembrane potential ($\Delta\psi_m$) is considered to be one of the central events in apoptotic death that leads to incapacitation of the mitochondria, release of cytochrome *c*, and activation of the caspase pathway, we tested the integrity of this signaling event using the fluorescent dye JC-1 as described elsewhere (44). Upon entering the mitochondrial negative transmembrane potential in healthy cells, JC-1 forms red fluorescent aggregates. When the transmembrane potential is low, as in many cells undergoing apoptosis, JC-1 exists as a monomer and produces green fluorescence. Consistent with this notion, green fluorescence was observed in dying cells after being transfected with AR siRNA 8 (as pointed by arrows in Fig. 5B, *c* and *d*), whereas living cells remained normal membrane potential (red fluorescence as pointed with arrowhead in Fig. 5B).

The presence of cytochrome *c* in the cytosol is a critical event required for the correct assembly of the apoptosome, subsequent activation of the executioner caspases, and induction of cell death (45). To evaluate the release of cytochrome *c*, cytosolic fraction of the cellular protein was collected 6 days after siRNA transfection. As shown in Fig. 6A, in parallel with the AR knocking down, cytochrome *c* was detected in the cytosolic fraction when AR siRNA 8 was transfected into cells. Meanwhile, the apoptosis hallmarker poly(ADP-ribose) polymerase cleavage fragment was also detected. Finally, the proteolytic processing of inactive procaspases, the essential component of the death pathway in many cells (21), and their catalytic activity were also analyzed. As shown in Fig. 6B, transfection with AR siRNA 31 into LNCaP cells induced significant reduction of procaspase-3, procaspase-6, and DFF45 (evidence for proteolytic activation or cleavage). Similar results were also seen when LAPC-4 or C4-2 cells were used (data not shown). Consistently, the catalytic activity of caspase 3/7 was significantly increased when AR siRNA 31 was used compared with negative control siRNA (Fig. 6C). Thus, these data clearly showed that the mitochondrial apoptotic mechanism is activated by the AR siRNAs.

Antiapoptotic Protein Bcl-xL Is Involved in AR-Mediated Cell Survival

Having shown the mitochondrial involvement in AR siRNA–induced cell death, we next focused on the Bcl-2 family members because they are the major regulators of mitochondrial function in the aspect of apoptosis by facilitating or inhibiting cytochrome *c* release to cytosol

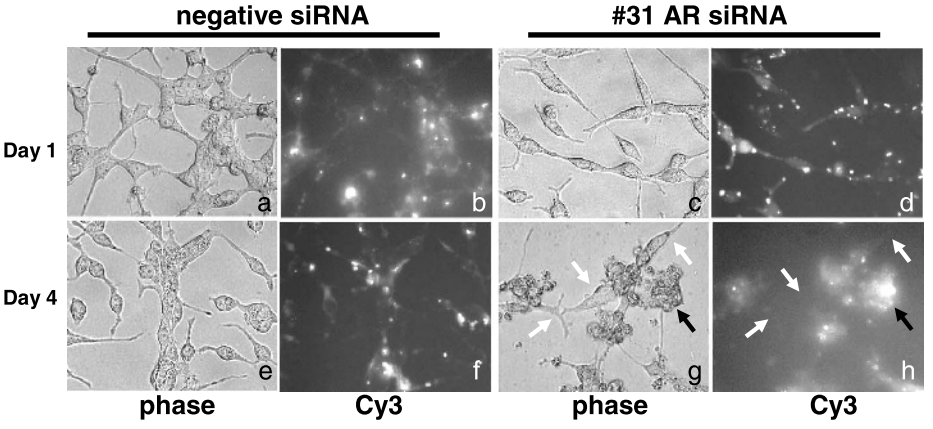

Figure 4. Visualization of the Cy3-labeled AR siRNA–induced cell death. LNCaP cells were seeded in six-well plates overnight and then transfected with Cy3-labeled siRNAs (10 nmol/L in the medium) as indicated. Cell death was monitored daily. Pictures were taken at days 1 and 4 after transfection. The Cy3-labeled siRNAs are seen as white dots in Cy3 (*b*, *d*, *f*, and *h*). In (*g*) and (*h*), white arrows indicate several living cells without the Cy3 labeling (negative transfection), whereas black arrows indicate a cluster of dying cells (round and detached) with strong Cy3 labeling (positive transfection).

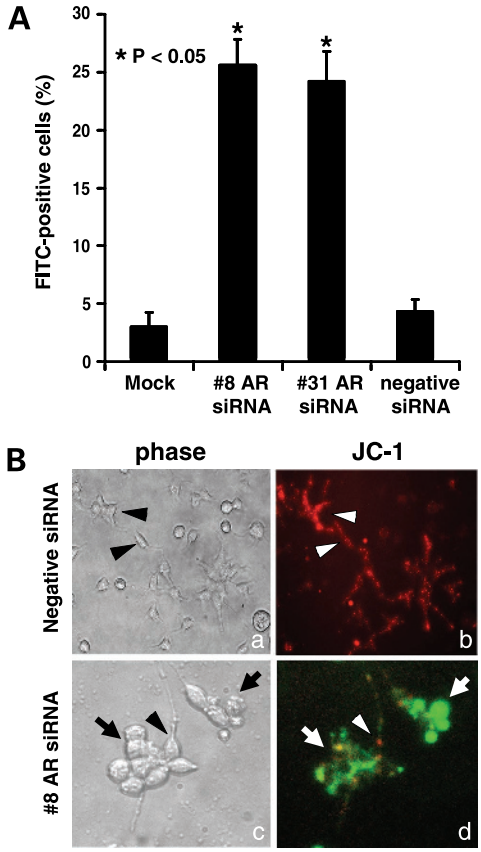

Figure 5. AR siRNA induces apoptotic cell death. **A,** after transfection with the siRNA duplexes (10 nmol/L in the medium) as indicated for 4 d, LNCaP cells were harvested and the change of the membrane phospholipid phosphatidylserine was determined using fluorescence-activated cell sorting for FITC-labeled cells as described in the text. Data are from two different experiments. **B,** following transfection with the siRNA duplexes (10 nmol/L in the medium) for 5 d, LNCaP cells were incubated with JC-1 (0.3 μg/mL) for 15 min at 37°C. Pictures were taken under a fluorescent microscope (magnitude ×200).

and subsequent assembly of an active apoptosome (22). These functions are promoted by the proapoptotic Bax or Bak and are inhibited by the antiapoptotic Bcl-2 and Bcl-xL. We determined whether protein expression of these Bcl-2 family members is altered after the AR siRNA transfection. Interestingly, we found that the protein level of the antiapoptotic member Bcl-xL dramatically decreased in the AR siRNA 8–transfected cells compared with the controls, whereas another antiapoptotic member, Bcl-2, and the proapoptotic members, Bax and Bak, remained unchanged (Fig. 7A). To better illustrate the relationship of Bcl-xL reduction with AR silencing, we conducted a time course experiment (Fig. 7B). The protein levels of Bcl-xL decreased in a time-dependent manner following the AR siRNA transfection; however, Bax protein remained consistent during the time course. These data indicate that AR silencing results in Bcl-xL reduction that might lead to an imbalance between the proapoptotic and antiapoptotic members of the Bcl-2 family that, in turn, triggers apoptosis.

To shed light onto the mechanistic basis underlying the response of Bcl-xL reduction to AR silencing, we also examined Bcl-xL expression at the mRNA level by RT-PCR assay. As shown in Fig. 7C, the Bcl-xL mRNA level decreased significantly after AR siRNA 8 transfection compared with the controls, indicating that the reduction of Bcl-xL protein after AR silencing is via a transcriptional mechanism.

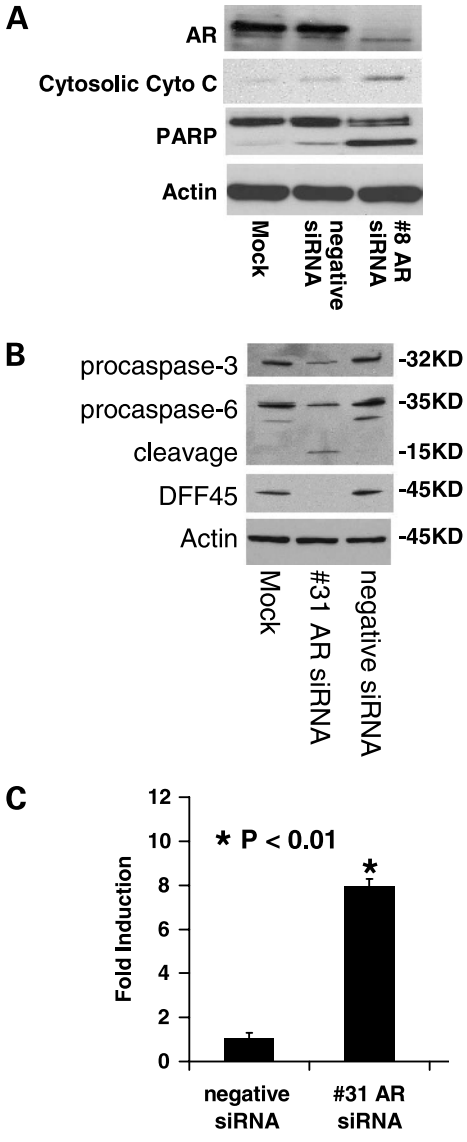

Figure 6. AR siRNA induces cytochrome *c* release, caspase activation, and cleavage of DFF45 and poly(ADP-ribose) polymerase. **A** and **B,** after 7 d of transfection with the siRNAs as indicated, LNCaP cells were harvested and the cytosolic occurrence of cytochrome *c*, proteolytic process of caspase-3 and caspase-6, and DFF45 and poly(ADP-ribose) polymerase cleavage were determined by Western blot. **C,** after 7 d of transfection with the siRNAs as indicated, LNCaP cells were washed with ice-cold PBS and then harvested. Caspase activity was measured as described in the text. *Columns,* mean value of relative activity from three independent experiments.

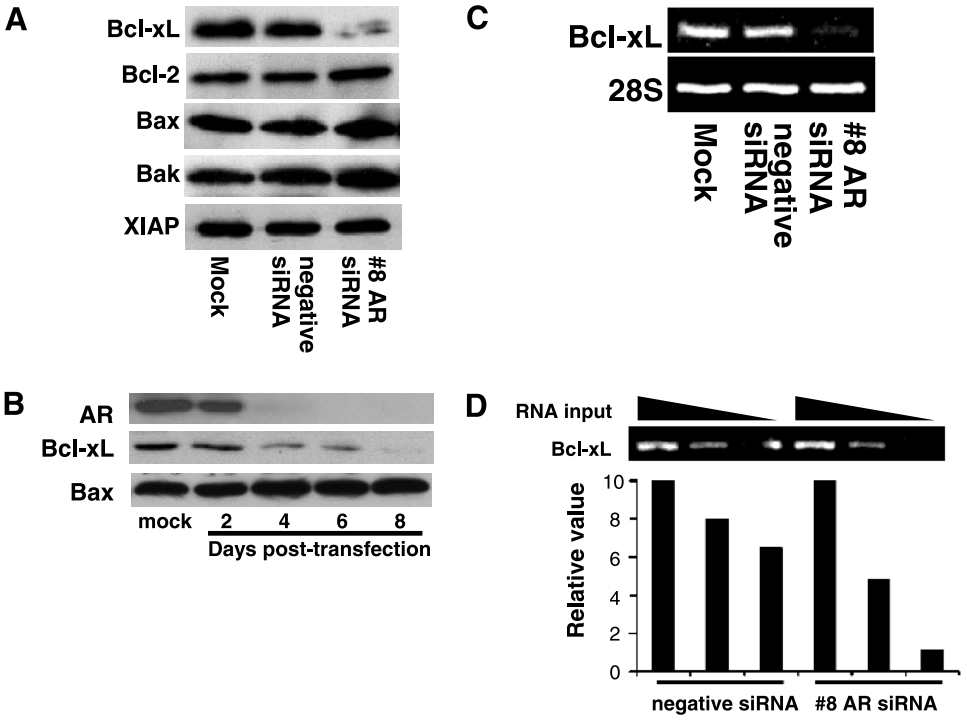

Figure 7. AR siRNA transfection leads to reduction of Bcl-xL expression. **A** and **B,** after transfection with AR siRNA 8 or negative control siRNA (10 nmol/L in the medium) as indicated, LNCaP cells were harvested on day 7 (**A**) or at each time point (**B**), and the protein levels of AR, Bcl-2, Bcl-xL, Bax, Bak, and XIAP were assessed by Western blot. Data was reproducible in three independent experiments. **C,** after transfection with the indicated siRNAs (10 nmol/L in the medium), LNCaP cells were harvested on day 7, the total RNA was isolated, and the Bcl-xl mRNA level was assessed by RT-PCR as described in the text. **D,** similar to (**C**), but a serial 10-fold dilution of the total RNA input was made for the first-strand cDNA synthesis in the RT-PCR assay. Relative band density in each lane was determined and graphed.

AR siRNA–Induced Apoptosis Was Partially Inhibited by Ectopic Bcl-xL Expression

In view of the antiapoptotic feature of Bcl-xL protein, we hypothesized that the AR promotes cellular survival by upregulating the *bcl-x* gene expression through a transcriptional mechanism in prostate cancer cells. Therefore, Bcl-xL expression will decrease if the AR is knocked down, which subsequently results in apoptosis due to an imbalance between the proapoptotic and antiapoptotic members of the Bcl-2 family. Thus, we wondered if an enforced Bcl-xL expression will protect cell from apoptosis while AR is silenced. To assess the protection effect of Bcl-xL protein, a stable LNCaP subline overexpressing human Bcl-xL protein controlled by a cytomegalovirus promoter (LNCaP/Bcl-xL) or a control subline with an empty vector (LNCaP/puromycin) were established. Consistent with the results obtained from the parental cells (Fig. 7A), exposure of those LNCaP subline cells to AR siRNA 8 resulted in a decrease of endogenous but not exogenous Bcl-xL protein (Fig. 8A, *lane 1* versus *lane 2*). Most significantly, as expected, enforced Bcl-xL expression partially inhibited cell death induced by AR siRNA transfection in LNCaP/Bcl-xL cells compared with the controls (Fig. 8A, *bottom*). These data showed that Bcl-xL is involved in AR-mediated survival of prostate cancer, and the reduction of Bcl-xL expression after AR silencing represents a mechanism for the AR siRNA–induced apoptosis.

In addition, while establishing a subclone for stable Bcl-xL expression in LNCaP cells, an unexpected clone [LNCaP subclone 11 (LN11)] was obtained, in which Bcl-xL expression was dramatically reduced for unknown reason, as confirmed by RT-PCR and Western blotting (Fig. 8B, *top* and *middle*). By taking advantage of this particular clone of LNCaP cell subline, we further tested the involvement of Bcl-xL in AR-mediated survival. Exposing LN11 subline cells to AR siRNA 8 resulted in a significant increase in AR siRNA–mediated cell death compared with the parental LNCaP cells and the untransfected controls (Fig. 8B, *bottom*), although the LN11 subline did not show a profound cell death without AR silencing. These data indicate that loss of Bcl-xL expression enhances AR siRNA–induced cell death, and multiple downstream factors, besides Bcl-xL, are mediating AR survival signal.

Discussion

In this report, we identified two siRNA duplexes that induce a strong AR silencing in prostate cancer cells. Most importantly, we found that siRNA-mediated AR silencing subsequently leads to a massive cell death through a mitochondrial apoptotic pathway. AR siRNA–induced apoptosis only occurs in prostate cancer cells that harbor an endogenous AR regardless of their androgen sensitivity. Further analyses showed that Bcl-xL expression is transcriptionally dependent on the AR in prostate cancer cells, and siRNA-mediated AR silencing results in a reduction of Bcl-xL expression that accounts partially for the apoptotic response because enforced Bcl-xL expression inhibited cell death after AR silencing. To the authors' knowledge, this is the first report showing AR involvement in Bcl-xL expression and apoptotic response to siRNA-mediated AR silencing in prostate cancer.

Our results are somewhat different from other approach-induced AR blockage as mentioned above (8, 13–15, 39), in which only cell arrest or reduced tumor growth, but no cell death, were reported. The plausible reason might be the difference in the extent of AR blockage or protein knockdown. For example, the AR-specific antibody might not totally block the AR function as used in a previous report (13) because the AR protein still exists in the cell. It is believed that the RNAi approach is more potent than the ribozyme (13) or antisense approach in terms of gene silencing (14, 15); therefore, our RNAi approach might have induced a more efficient knocking down of the AR protein than the former approach of AR ribozyme or antisense oligonucleotides. In addition, current experiences in the field of RNAi technology showed that the siRNAs targeted to different regions of a gene transcript may not function equally (31), which may be responsible for the different findings between our results and others (8, 39). Finally, the strategies in the experimental condition used between ours and other groups (8, 39) might also account for the different outcome.

It has been shown that androgen or other factors as critical survival stimuli play an important role via the AR in prostate cancer progression. Although AR-dependent functional repression of FKHR and related FOXO forkhead proteins was reported as a possible pathway (18), the survival pathway of AR-dependent mechanism is not clear. PI3K-Akt is a major cellular survival factor that is negatively regulated by the PTEN phosphatase (46). In LNCaP cells, Akt is constitutively active due to PTEN mutational inactivation (47), whereas LAPC-4 cells maintain a wild-type PTEN (48). Here, we observed that AR siRNA induced cell death in both of the cell lines, suggesting that the AR-dependent survival pathway is via an AKT-independent mechanism, which was also proposed by previous reports (49, 50). In addition, we observed the apoptotic response from those native AR-harboring cells (RWPE-1, LAPC-4, LNCaP, CWR22Rv1, and C4-2), but not from the AR-null PC-3 or its subline PC-3/AR cells, which was reconstituted with an exogenous *AR* gene. These findings indicate that the AR is a critical survival factor for prostate epithelium-derived cells and remains as an important survival factor even in those hormone-refractory cancer cells, although they might have developed additional survival mechanism. However, the AR-null prostate cancer cells already escaped from the AR-regulated survival control.

The Bcl-2 family proteins reside immediately upstream of mitochondria and function as either death antagonists

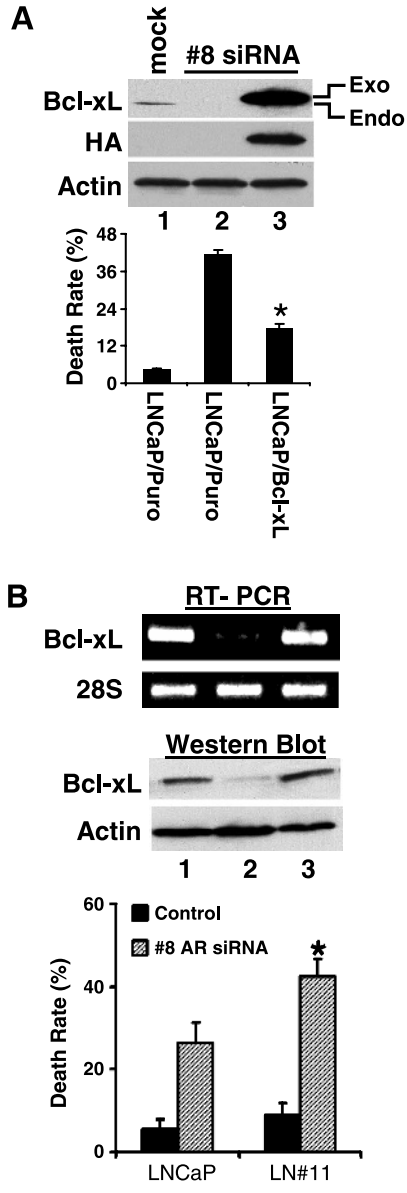

Figure 8. **A,** ectopic enforced expression of *bcl-xl* gene inhibits AR siRNA–induced cell death. LNCaP/puromycin and LNCaP/Bcl-xL cells were transfected with AR siRNA 8 for 7 d and the expression level of endogenous/exogenous *bcl-xl* gene was determined by Western blot. Because the exogenous Bcl-xL protein has a HA tag, the membrane was reprobed with anti-HA antibody to show the exogenous Bcl-xL protein. Actin blot served as loading control. The cell death rate (blue cells versus blue plus white cells) was determined individually by trypan blue exclusion assay. The asterisk indicates a significant difference ($P < 0.05$) between LNCaP/puromycin versus LNCaP/Bcl-xL cells after AR siRNA 8 transfection. Data are from three independent experiments. **B,** loss of Bcl-xL expression lead to a significant increase of AR siRNA–induced cell death. *Top,* the parental LNCaP cells (*lane 1*), LNCaP subline LN11 (*lane 2*), and a stable subclone bearing an empty vector (*lane 3*) were exponentially grown and harvested. Total RNA was isolated and Bcl-xL mRNA levels were determined by RT-PCR and 28S gene served as internal control for the RT-PCR assay. Cellular proteins were extracted and Bcl-xL protein levels were assessed by Western blot. Antiactin blot served as loading control. Data are from two separate experiments. *Bottom,* cells were transfected with negative siRNA (*black columns*) or AR siRNA 8 (*gray columns*) at 10 nmol/L in the culture medium supplied with 2% charcoal-stripped FBS. Cell death rate [dying cells versus (dying plus living cells)] was determined 5 d later by trypan blue exclusion assay as described earlier. The asterisk indicates a significant difference ($P < 0.05$) between LN11 versus the parental LNCaP cells.

or agonists. The ratio of death antagonists to agonists determines how a cell responds to an apoptotic signal. Like Bcl-2, Bcl-xL is another major apoptotic antagonist and its expression is mainly regulated through transcriptional mechanisms (21, 22). The *bcl-x* promoter contains consensus motifs for a number of transcription factors, including Sp1, activator protein-1, Oct-1, Ets, Rel/NF-κB, signal transducers and activators of transcription (STAT), and GATA-1, in which three transcription factor families, STATs, Rel/NF-κB, and Ets family, have been shown to play an important role in the regulation of the *bcl-x* gene expression (26, 27). Recently, other steroid hormone receptors, including receptors for glucocorticoid and progesterone, have been reported to bind to the mouse *bcl-x* promoter (51, 52). In this report, our data suggest that the AR is involved in the transcriptional regulation of *bcl-x* gene expression, although the underlying mechanism is under further investigation by our group.

Recently, siRNA-mediated IFN response has emerged as a big concern regarding the use of siRNA in mammalian cells (40, 53). In our system, we also observed the similar response in which the transcriptionally made siRNAs induced more significant IFN response than the chemically synthesized ones.[5] However, similar responses were observed in all of those prostate cancer cells used in our study, indicating that the apoptotic effect of AR siRNA in the AR-harboring cells is independent of the IFN-related effect. Moreover, it was reported that only tumor necrosis factor-α, but not IFN, down-regulates Bcl-xL expression (54), suggesting that the reduction of Bcl-xL protein is not due to the siRNA-triggered IFN response.

In conclusion, our results showed for the first time that knocking down the AR protein by a siRNA duplex induces apoptosis in native AR-positive prostate cells regardless their hormone sensitivity. The apoptotic response induced by the AR siRNAs is partially due to reduction of Bcl-xL expression because enforced Bcl-xL expression inhibits AR siRNA-induced cell death. Currently, the underlying mechanism for AR-mediated up-regulation of the *bcl-x* gene is under further investigation. The siRNA-mediated AR silencing may be implicated as a novel approach in the future for curing the hormone-refractory prostate cancers that are currently considered as a condition with no cure.

Acknowledgments

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Serum/glucocorticoid-induced protein kinase-1 facilitates androgen receptor-dependent cell survival

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Androgen receptor (AR) is a critical factor in the development and progression of prostate cancer. We and others recently demonstrated that eliminating AR expression leads to apoptotic cell death in AR-positive prostate cancer cells. To understand the mechanisms of AR-dependent survival, we performed a genome-wide search for AR-regulated survival genes. We found that serum/glucocorticoid-induced protein kinase-1 (SGK-1) mRNA levels were significantly upregulated after androgen stimulation, which was confirmed to be AR dependent. Promoter analysis revealed that the AR interacted with the proximal and distal regions of the *sgk1* promoter, leading to sg*k-1* promoter activation after androgen stimulation. Functional assays demonstrated that SGK-1 was indispensable for the protective effect of androgens on cell death induced by serum starvation. SGK-1 overexpression not only rescued cells from AR small-interfering RNA (siRNA)-induced apoptosis, but also enhanced AR transactivation, even in the absence of androgen. Additionally, SGK-1 siRNA reduced AR transactivation, indicating a positive feedback effect of SGK-1 expression on AR-mediated gene expression and cellular survival. Taken together, our data suggest that SGK-1 is an androgen-regulated gene that plays a pivotal role in AR-dependent survival and gene expression.
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Androgens play a critical role not only in the physiological development of the prostate but also in the genesis of prostate cancer (reviewed by Heinlein and Chang[1]). These hormones regulate the activity of the androgen receptor (AR), a ligand-activated transcription factor and a member of the nuclear receptor superfamily.[2] Hormone–receptor complexes bind to certain hormone responsive DNA sequences proximal to target genes and regulate their expression.[1–3]

It is well demonstrated that castration in rodent models or androgen ablation in cell culture system causes a rapid apoptotic response in the prostate-derived epithelial cells.[4,5] Based on these phenomena, androgen ablation therapy has been used in clinical management of prostate cancer patients.[6] However, the disease progresses to a more aggressive androgen-independent stage (termed 'hormone-refractory' because the tumor is resistant to androgen ablation therapy).[7] The etiology of hormone-refractory progression may have various molecular causes, but a critical role of the AR has emerged.[8–11] We and others have recently showed that eliminating AR expression led to a profound apoptotic response in AR-positive prostate cancer cells, suggesting that the AR is essential for cellular survival in prostate cancers.[12–15] However, the downstream effectors that facilitate AR-dependent survival are not fully elucidated.

Serum and glucocorticoid-induced protein kinase-1 (SGK-1) belongs to the 'AGC' subfamily of protein kinases, including protein kinase A, G, and C.[16] There are two more isoforms of SGK-1 in mammalian cells, namely SGK-2 and -3. They are evolutionally conserved and share an 80% homology in the catalytic domains.[17] Particularly, SGK-1 shares about 54% identity of its catalytic domain with a major cellular survival factor protein kinase B (PKB, also called Akt), indicating a functional similarity. In fact, like Akt, SGK-1 is activated by phosphorylation through a phosphatidylinositol 3-kinase (PI3K)-dependent signal pathway.[18,19] However, unlike other protein kinases in AGC family, such as Akt, SGK-1 but not SGK-2 or -3 is regulated and activated mainly through a transcriptional mechanism by extracellular stimuli. Its promoter contains several consensus sequences for transcription factors that can be activated by a very large spectrum of stimuli besides glucocorticoid and serum.[19,20] Upon induction, it acts as a cell survival factor in different situations and is also able to regulate numerous membrane transporters and channel proteins.[18,19]

In the studies reported here we identified a downstream effecter pathway responsible for AR-dependent survival. We found that expression of SGK-1 but not other isoforms or Akt was upregulated by androgen stimulation. Further analysis confirmed that SGK-1 is an androgen-induced gene via an

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Keywords: SGK-1; androgen receptor; prostate cancer; survival; gene regulation
Abbreviations: AAV, adeno-associated virus; AR, androgen receptor; ARHP, AR hairpin; cDNA, complementary DNA; ChIP, chromatin immunoprecipitation; cFBS, charcoal-stripped FBS; CMV, cytomegalovirus; CSDX, Casodex; FBS, fetal bovine serum; GRE, glucocorticoid response element; IκB-α, I kappa B-alpha; IKKβ, IκB kinase β; NF-κB, nuclear factor-κB; PB, probasin; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PI3K, phosphoinositide 3–kinases; PKB, protein kinase B; PSA, prostate-specific antigen; RT, reverse transcription; SE, standard error; SEAP, secreted alkaline phosphatase; SGK-1, serum/glucocorticoid-induced protein kinase-1; siRNA, small-interfering RNA; SRE, steroid response element; TBS-T, Tris-buffered saline/Tween-20
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AR-dependent transcriptional mechanism. Functional assays demonstrated that SGK-1 is indispensable from AR-mediated survival. SGK-1 overexpression enhances AR-mediated gene expression and reduces cell death induced by AR small interfering RNA (siRNA), suggesting a positive feedback effect of androgen-induced SGK-1 expression on AR-dependent gene expression and survival.

Results

AR-dependent SGK-1 expression after androgen stimulation. We and others recently demonstrated that the AR is essential for cellular survival of AR-positive prostate cancer cells.[12–15] To better understand the mechanism of AR-dependent survival, we performed a genome-wide search for AR-regulated survival genes using complementary DNA (cDNA) microarray analysis. Prostate cancer LNCaP cells were serum-starved for 48 h, and were then treated with the solvent or a synthetic androgen R1881 (1.0 nM) for 8 h. The Affymetrix human genome U133A chips were used to analyze the alterations of androgen-induced gene expression. SGK-1 was identified as one of the genes significantly upregulated by androgen stimulation compared to the solvent control (Table 1). Other SGK isoforms and the closest relative protein kinases Akt-1 or -3 remained unchanged after androgen stimulation. These data were supported by a recent publication from another group using the same strategy, which showed an 8.1-fold increase of SGK-1 expression by androgen treatment.[21]

We confirmed the microarray data using additional methods, including conventional reverse transcription (RT)-polymerase chain reaction (PCR) and real-time RT-PCR assays for mRNA expression, as well as western blot for protein expression. LNCaP cells were serum-starved for 48 h and then treated with R1881 for 8 h (mRNA analysis) or 24 h (protein analysis) under serum-free conditions. To test for AR dependency, the AR antagonist bicalutamide (Casodex™, CSDX) were used to pre-treat the cells before R1881 addition. A well-known AR-dependent gene prostate-specific antigen (*PSA*) was included in the analysis as a positive control. Ribosomal 28S RNA was used as a loading control for RT-PCR experiments. As illustrated in Figure 1a, SGK-1 expression was not detectable under the conditions of serum starvation. Similar to *PSA*, SGK-1 expression was dramatically increased by R1881 stimulation. The R1881 effect was blocked by CSDX pretreatment, indicating that androgen-induced SGK-1 expression is AR dependent. AR-mediated SGK-1 expression was further confirmed using real-time RT-PCR (Figure 1b) and Western blot (Figure 1c). However, there was no significant alteration of Akt-1 expression after R1881 treatment, consistent with the data from cDNA microarray analysis (Table 1). These results suggest that SGK-1 is an androgen-inducible gene via AR-dependent transcription in prostate cancer cells.

As mentioned earlier, AR gene silencing results in apoptotic cell death.[12–15] Since the survival kinase SGK-1 is an

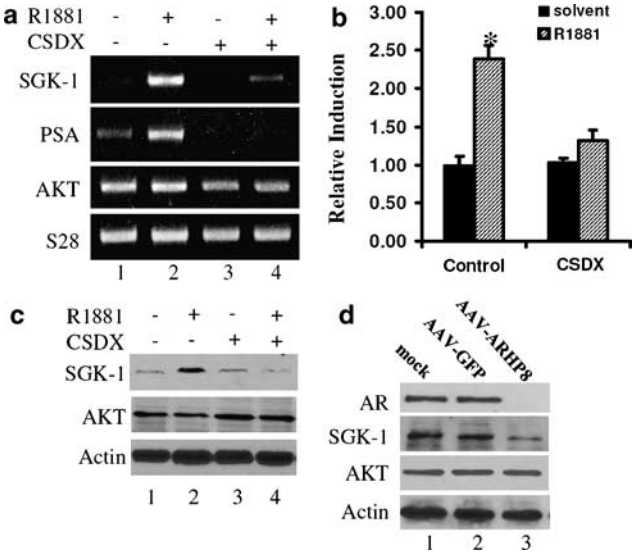

Figure 1 Androgen receptor regulates SGK-1 expression. (**a**, **b**) LNCaP cells were serum-starved for 48 h and then pretreated with the anti-androgen bicalutamide (CSDX) at 10 μM or the solvent for 30 min. Cells were then stimulated with the synthetic androgen R1881 at 1.0 nM in serum-free media for 8 h before harvest. Total RNA was extracted by TriZol™-based protocol. Gene expression at mRNA level was assessed by RT-PCR (**a**) or real-time RT-PCR (**b**). For RT-PCR, human *psa* gene served as positive control of androgen action while 28S ribosomal gene was used as negative control. A set of the representative data was shown. For the real-time RT-PCR, the expression level of *sgk-1* gene was normalized against *β-actin* expression level before the relative value was calculated compared to the solvent control (set as value 1.0). Data represent three separate experiments. Error bar represents the standard error (S.E.), and the asterisk indicates a significant difference ($P<0.05$, *t*-test) between R1881 treatment and the solvent control. (**c**) After bicalutamide pretreatment as above, cells were treated with R1881 (1.0 nM) for 24 h and were then harvested. Whole cellular protein extracts were used for Western blot to assess protein expression levels of SGK-1 and Akt. Membranes were reprobed with anti-Actin as the loading control. (**d**) LNCaP cells were infected with AAV-GFP or AAV-ARHP8 at 1.0×10^3 viral particles per cell in serum-reduced media (2% cFBS) for 5 days. A mock infection was included as negative control. Whole cell lysates were used for Western blot to determine protein levels of AR, SGK-1, and Akt. Anti-Actin blot served as a loading control. Data represent two separate experiments

Table 1 cDNA microarray analysis of androgen-stimulated gene expression

Gene symbol	Probe identification	Fold induction	Control signal	Treatment signal
SGK-1	201739_at	***6.62***	71.9	475.8
SGK-2	213837_at	1.09	139.2	152
SGK-3	220038_at	1.72	173.8	298.5
AKT-1	207163_s_at	0.79	1,496	1192
AKT-3	219393_s_at	1.82	96.1	175.1
GAPDH	212581_x_at	1.01	25 327	25 767

AR-regulated gene, we asked if AR silencing led to SGK-1 downregulation, which consequently led to apoptotic cell death. To test this hypothesis, we measured SGK-1 protein expression after AR gene silencing. To introduce the AR siRNA more efficiently, we created an adeno-associated virus (AAV) that carries a hairpin-structured AR siRNA (AAV-ARHP8) expression cassette based on the AR siRNA #8 sequence used in our previous work.[12] LNCaP cells were infected with the AAV-ARHP8 viruses or a control empty virus (AAV-GFP) for 5 days in androgen withdrawal media (2% charcoal-stripped fetal bovine serum (FBS), cFBS). As shown in Figure 1d, AAV-ARHP8 infection resulted in a complete loss of AR expression. In parallel, the protein level of SGK-1 but not Akt was reduced dramatically in AAV-ARHP8-infected cells compared to the control cells. These results confirmed our hypothesis that SGK-1 expression is regulated by the AR through either androgen-dependent or -independent mechanisms, such as growth factors and cytokines.[7] The detailed pathways are under further investigation by our group.

AR activates SGK-1 promoter following androgen stimulation. The AR, together with the receptors of glucocorticoid, progesterone and mineralocorticoid, are closely related members (class I) in the nuclear receptor superfamily.[22] They all recognize similar response elements (steroid response element, SRE), which are organized as inverted repeats of 5′-TGTTCT-3′-like sequences with a three-nucleotide spacer.[23] We and others have shown that SGK-1 expression is regulated by glucocorticoid via an imperfect glucocorticoid response element (GRE) on the *sgk1* promoter.[16,24,25] Therefore, we tested if androgen activates *sgk1* promoter. We used a luciferase reporter (F4R4-Luc) that was described in our previous publication.[24] This reporter uses a $-3142/+117$ fragment relative to the transcription starting site of human *sgk1* gene as the promoter (Figure 2a). LNCaP (mutant AR) and LAPC-4 (wild-type AR) cells were transfected with the F4R4-Luc and the control reporter constructs. Following serum starvation for 24 h and bicalutamide pretreatment for 45 min, cells were stimulated with R1881 for 24 h in androgen withdrawal media

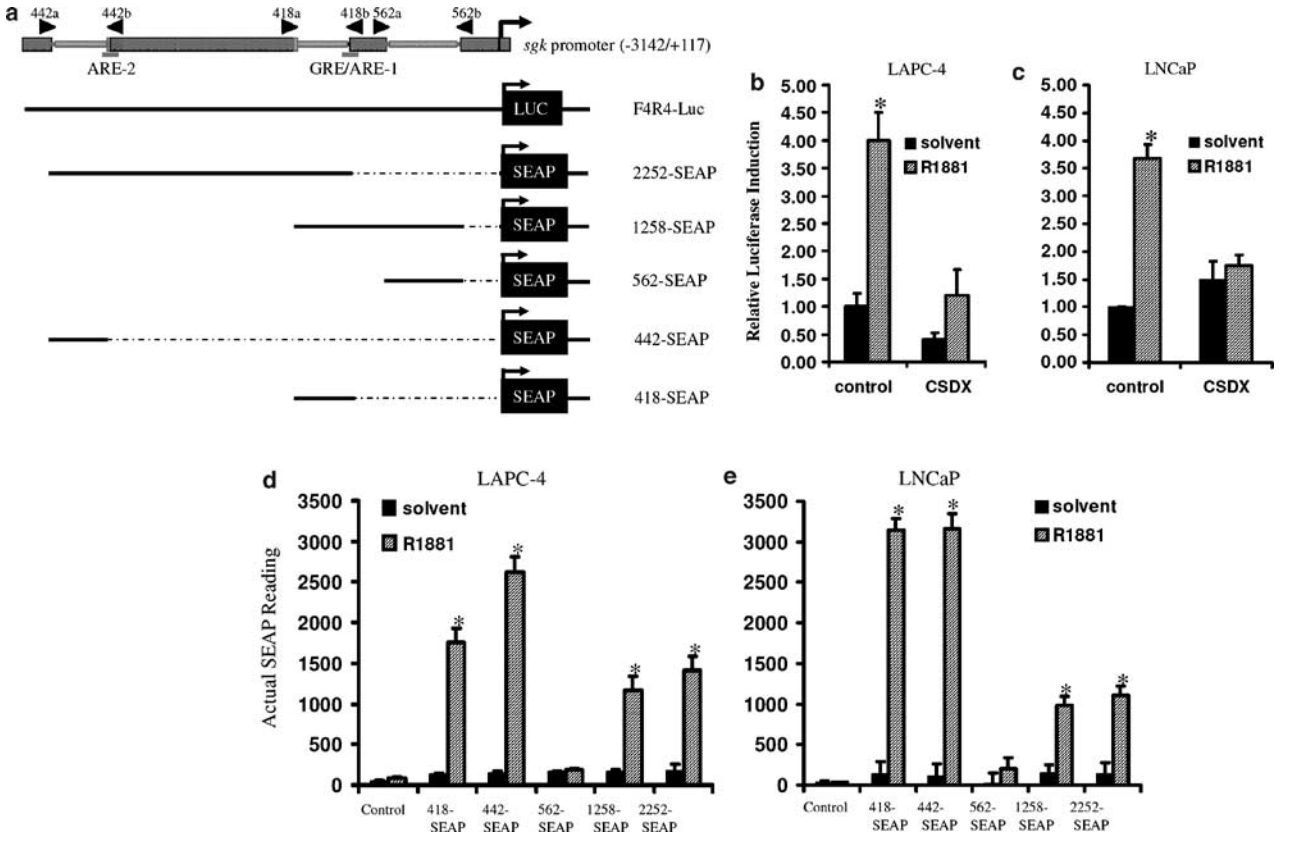

Figure 2 Androgen receptor transactivates *sgk-1* promoter. (**a**) Schematic illustration of *sgk-1* promoter, the GRE/ARE-1 and the putative secondary ARE-2 location, ChIP primer binding sites, and structure of SEAP gene reporters driven by different fragments of *sgk-1* promoter. (**b**, **c**) LAPC-4 or LNCaP cells were transfected with *sgk-1* promoter-driven luciferase reporter (pGL3-SGK1/F4R4-LUC) together with an internal control reporter pCMV-SEAP. After serum starvation, cells were pretreated with bicalutamide (CSDX, 10 μM) or the solvent, and were then stimulated with R1881 (1.0 nM) for 24 h in 5% cFBS-containing media. Culture supernatants were collected for SEAP assay and cellular proteins were used for luciferase assay. Luciferase activities were normalized against the corresponding protein concentration and SEAP activity before the relative fold induction was calculated compared to the solvent control. (**d**, **e**) Cells as indicated were transfected with different SEAP reporter constructs, and a mock transfection was included with transfection reagent only. Following serum starvation, cells were stimulated with R1881 (1.0 nM) or the solvent for 24 h in 5% cFBS-containing media. Culture supernatants were collected for SEAP assay and cells were harvested for protein assay. SEAP activities were normalized against their corresponding cellular protein levels. Data represent three different experiments, and the error bar indicates S.E. The asterisk indicates a significant difference ($P<0.05$, *t*-test) between R1881 treatment and the solvent control

(5% cFBS). As shown in Figure 2b, R1881 significantly increased the reporter activity in both cell lines, and bicalutamide pretreatment abolished R1881-stimulated F4R4-LUC activation. These data confirmed that androgen activates SGK-1 promoter via the AR.

To narrow down the position of AR responsive elements within the *sgk1* gene promoter, we generated five different secreted alkaline phosphatase (SEAP) reporter constructs using different fragments from the *sgk1* gene promoter. These fragments were PCR-amplified from LNCaP cell genomic DNA, as illustrated in Figure 2a. The proximal promoter (−1548/−290) was termed as 1258-SEAP, which contains the GRE motif (−1147/−1133) identified previously. Using this proximal promoter, we constructed two truncated promoters, named as 418-SEAP (−1548/−1130 with the GRE motif) and 562-SEAP (−852/−290 without the GRE motif). The distal promoter 2252-SEAP (−3382/−1130) and its truncated one 442-SEAP (−3382/−2940) contain several putative SRE motifs, as suggested by a computer-based inspection.[19] The androgen responsiveness of these reporters was analyzed in LNCaP and LAPC-4 cells. As shown in Figure 2d and e, R1881 treatment significantly activated the reporters driven by the proximal (1258-SEAP) and distal (2252-SEAP) promoter fragments in both cell lines. This responsiveness was further increased in those truncated fragments of the proximal (418-SEAP) and the distal (442-SEAP) promoters. This increased response to androgen stimulation from the truncated distal promoter (442-SEAP) compared to the distal (2252-SEAP) promoter indicated the existence of a potential secondary ARE motif (ARE-2) within the far distal region of the *sgk1* gene promoter, as illustrated in Figure 2a. In contrast, the truncated minimum promoter (562-SEAP) lacking the GRE motif did not response to R1881 stimulation, thereby confirming that the proximal GRE (hereafter termed as ARE-1) is required for androgen-induced SGK-1 expression.

Then, we went on to determine if the AR physically interacts with the ARE-containing regions on the *sgk1* gene promoter we had indicated using the reporter assays. Serum-starved LNCaP cells were pretreated with CSDX followed by R1881 stimulation. Chromatin immunoprecipitation (ChIP) assays were used to analyze the AR interaction with genomic DNA *in vivo*. We chose to test three different regions on the *sgk1* promoter: (a) the close proximal region, (b) the GRE/ARE-1 region, and (c) the far distal region, as illustrated in Figure 2a. Without R1881 stimulation, no interaction was detected between the AR and any promoter regions (Figure 3, lane 1). R1881 stimulation led to a clear signal of the AR interaction with the regions that contain either the GRE/ARE-1 motif or the ARE-2 motif in the far distal fragment. Consistent with the data from 562-SEAP reporter assay that showed no androgen responsiveness, no interaction was detected between the AR and the proximal promoter region. These results were in agreement with the conclusion from the reporter gene assays, and show that the AR regulates SGK-1 expression by binding to two sites in the promoter region of the *sgk-1* gene. The secondary ARE-2 motif resides in the far distal region of the *sgk1* gene promoter, as proposed by others previously.[19]

SGK-1 is required for AR-dependent survival. Similar to Akt, SGK-1 has been shown to be a cellular survival factor.[26–29] Therefore, we examined the role of SGK-1 in AR-dependent survival of prostate cancer cells. First, we tested if eliminating SGK-1 expression induces apoptosis. LAPC-4 cells were transfected with SGK-1 siRNA or a control siRNA, and were then kept in androgen withdrawal media

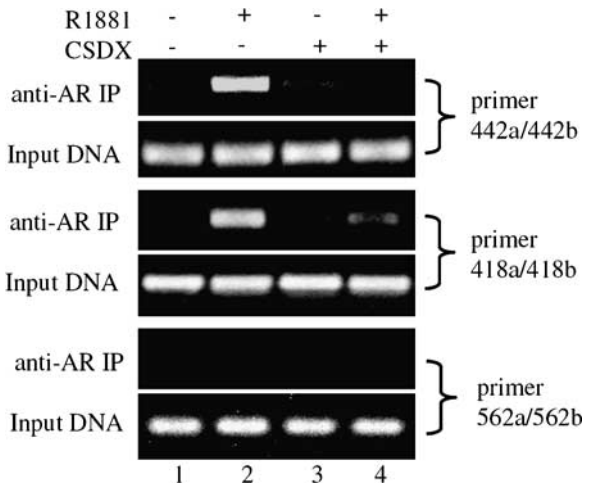

Figure 3 Androgen receptor interacts with the ARE locus on the *sgk1* promoter. LNCaP cells were serum-starved for 2 days and were then pretreated with bicalutamide (CSDX, 10 μM). After stimulation with R1881 (1.0 nM) or the solvent in 2% cFBS-containing media, cells were subjected to ChIP assay, as described.[38] Anti-AR immunoprecipitated chromatin DNA were amplified with three different primer sets, and 10% of whole cellular genomic DNA were used as PCR templates as the positive control. Data represent two different experiments

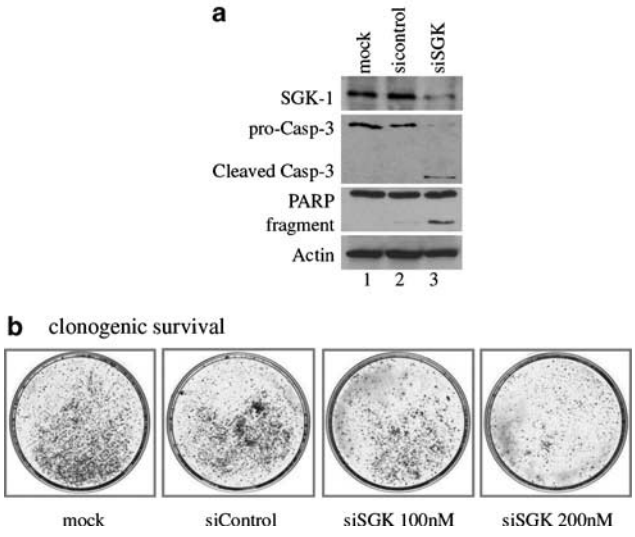

Figure 4 SGK-1 siRNA induces apoptotic cell death. (**a**) LAPC-4 cells were transfected with SGK-1 siRNA or the negative control siRNA at a final concentration of 100 nM in media. A mock transfection was included as a negative control. Cells were cultured in androgen withdrawal media (2% cFBS) for 5 days. Whole cell lysates were then used for western blot with antibodies as indicated. Actin blot served as the loading control. Data represent three separate experiments. (**b**) LNCaP cells were transfected with the negative siRNA or SGK-1 siRNA at different concentrations as indicated in androgen withdrawal media (2% cFBS). After 7 days, survived colonies were stained and photographed. Data represent two separate experiments

(2% cFBS) for 5 days. As shown in Figure 4a, transfection with the SGK-1 siRNA but not the control siRNA resulted in a dramatic loss of SGK-1 expression. In parallel, cell death was observed (cell round-up and floating in media under inverted microscope, data not shown) in cells transfected with SGK-1 siRNA but not with the control siRNA. After cell harvesting, we examined two apoptotic hallmarks, caspase-3 activation and PARP cleavage, to determine if SGK-1 siRNA-induced cell death was an apoptotic response. As expected, caspase-3 activation and PARP cleavage were observed in SGK-1 siRNA-transfected cells but not in the controls (Figure 4a). These data suggest that SGK-1 elimination leads to apoptosis, which is supported by previous reports.[26–29]

Next, we confirmed that SGK-1 siRNA could induce apoptosis in another prostate cancer cell line. LNCaP cells were tested using a clonogenic assay. After transfection with the control or SGK-1 siRNA, cells were cultured in the same condition (2% cFBS) for 7 days. Surviving cells were visualized by crystal violet staining. As shown in Figure 4b, the surviving cell fraction was remarkably reduced in cells transfected with SGK-1 siRNA, especially in cells transfected with higher dose of SGK-1 siRNA, compared to the control siRNA-transfected cells. These data confirmed that SGK-1 is important for prostate cancer cell survival.

Since androgen was found to regulate SGK-1 expression, we asked if SGK-1 is required for androgen-induced survival in prostate cancer cells. LAPC-4 cells were transfected with SGK-1 siRNA or the control siRNA and cell death was initiated by serum starvation in the presence or absence of R1881. As shown in Figure 5a, in mock and control siRNA-transfected cells, serum starvation resulted in a moderate level of cell death. The death response was dramatically increased in cells transfected with SGK-1 siRNA. As expected, R1881 addition significantly reduced serum starvation-induced cell death in mock and control siRNA-transfected cells but not in SGK-1 siRNA-transfected cells. These results indicate that SGK-1 is important in androgen-induced survival.

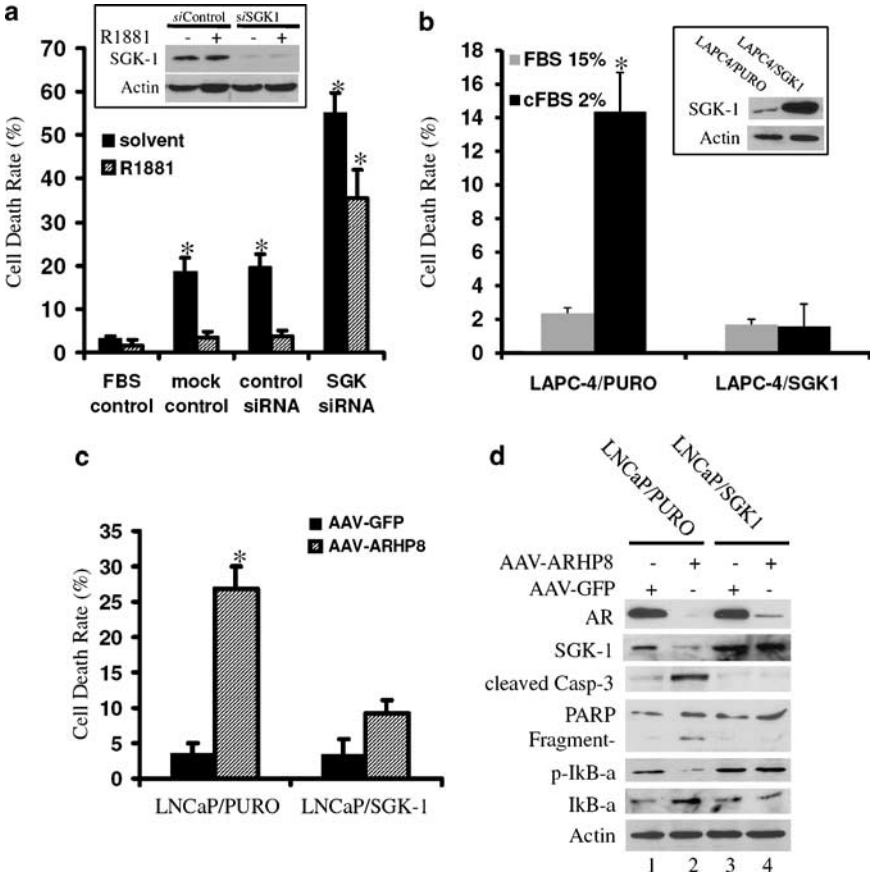

Figure 5 SGK-1 facilitates androgen receptor-dependent survival. (**a**) LAPC-4 cells were transfected with the control siRNA or SGK-1 siRNA (100 nM), and a mock transfection was also included. Transfected cells were cultured in serum-free media, and the untransfected control cells were cultured in a full culture media with serum (15% FBS) in the presence or absence of R1881 (1.0 nM) for 5 days. Cell death rates were assessed with trypan blue exclusion assay. The inserted immunoblotting panels show the efficacy of SGK-1 siRNA and the antibodies as indicated on the left-hand side. (**b**) LAPC-4 stable sublines LAPC-4/SGK1 and LAPC-4/PURO were cultured in either full growth media (15% FBS) or androgen-withdrawal media (2% cFBS) for 10 days. Cell death rates were determined as above (**a**) by trypan blue exclusion assay. The inserted immunoblotting panels show the level of SGK-1 overexpression in the sublines, and the antibodies as indicated on the left-hand side. (**c**) LNCaP stable sublines LNCaP/SGK1 and LNCaP/PURO were infected with the AAVs as indicated at 1.0×10^3 viral particles per cell in serum-free media for 7 days. Cell death rates were determined by trypan blue exclusion assay (**a**, **b**) and cellular proteins were used for western blot analysis with the antibodies as indicated on the left-hand side (**d**). Data represent three separate experiments. For panel **a**, **b**, and **c**, error bar indicates S.E. and the asterisk indicates a significant difference ($P < 0.05$, *t*-test) compared to *FBS control* (**a**, **b**) or AAV-GFP infected cells (**c**)

Subsequently, we tested to determine if overexpression of SGK-1 would protect cells from cell death induced by androgen withdrawal. An LAPC-4 subline was established that overexpresses SGK-1 (LAPC-4/SGK1). A companion control line (LAPC-4/PURO) harbors an empty vector. Cells were kept in complete culture media (15% FBS) or in androgen-withdrawal condition (2% cFBS). Cell death was determined at day 10. As shown in Figure 5b, androgen withdrawal induced a significant cell death in the control LAPC-4/PURO cells but not in LAPC-4/SGK1 cells, indicating again that SGK-1 is a potent survival factor in protecting against cell death after androgen withdrawal.

We showed above (Figure 1d) that AR silencing led to downregulation of SGK-1 in LNCaP cells. To understand the role of SGK-1 in AR-dependent survival, we tested whether SGK-1 overexpression could rescue cells from AR siRNA-induced cell death.[12,13] An LNCaP subline was established that stably overexpresses SGK-1 (LNCaP/SGK1), and its control subline was generated with an empty vector (LNCaP/PURO). Cell death was monitored after infecting the cells with AAV-ARHP8 virus under serum-free conditions. The empty virus AAV-GFP served as the control. The cell death rate was assessed 7 days later. As expected, AAV-ARHP8 virus induced a significant cell death compared to the control virus in the control LNCaP/PURO cells (Figure 5c), a finding consistent with our previous publication.[12] However, AAV-ARHP8 virus-induced cell death was significantly reduced in LNCaP/SGK1 cells. Caspase-3 activation and PARP cleavage were also largely suppressed in LNCaP/SGK1 cells compared to the LNCaP/PURO control cells (Figure 5d). Together with the data shown in Figure 1d, these results indicate that SGK-1 plays a pivotal role in AR-dependent survival.

Recently, SGK-1 has been found to promote cellular survival by activating Iκ-B kinase β (IKK-β). This effect leads to increased I kappa B-alpha (IκB-α) phosphorylation and nuclear factor-κB (NF-κB)-dependent survival,[28] and also suppresses FOXO3 transactivation.[29] Therefore, we tested to see if IκB-α phosphorylation was altered after AR silencing or SGK-1 overexpression. As shown in Figure 5d, IκB-α phosphorylation was largely reduced in LNCaP/PURO cells after AR silencing compared to the control (lane 2 *versus* lane 1), which was associated with SGK-1 downregulation. In contrast, higher levels of IκB-α phosphorylation were observed in LNCaP/SGK1 cells compared to the control LNCaP/PURO cells, even after AR silencing (lanes 3 and 4). On the other hand, the total level of IκB-α protein was increased in LNCaP/Puro cells after AR silencing, reflecting a reduced IκB-α degradation due to a lack of phosphorylation.[30] These data suggest that NF-κB activation after SGK-1-induced IκB-α phosphorylation and degradation might be a part of the mechanisms involved in AR-dependent SGK-1-mediated survival in prostate cancer cells.[31]

SGK-1 modulates AR-mediated gene expression. Similar to the regulation of Akt, *sgk-1* also can be activated via a PI3K-dependent mechanism.[32] We previously showed that PI3K activity (but not Akt) is required for AR-mediated gene expression.[33] Therefore, we determined whether SGK-1 modulates AR transactivation. First, we tested whether SGK-1 overexpression could enhance androgen-stimulated AR transactivation. LNCaP and LAPC-4 cells were transfected with an AR responsive gene reporter (ARE-LUC) together with increasing amounts of constructs expressing SGK-1. After serum starvation, cells were treated with R1881 or the solvent for 24 h. As shown in

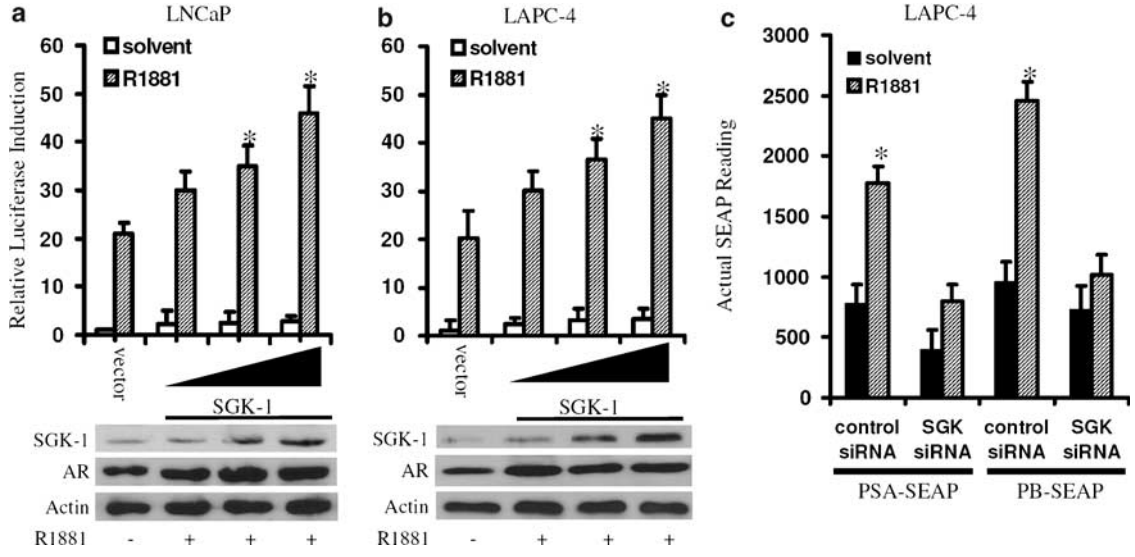

Figure 6 SGK-1 modulates androgen receptor-mediated gene expression. (**a**, **b**) LNCaP or LAPC-4 cells were placed on six-well plates overnight and were transfected with an androgen response gene reporter ARE-LUC (1.0 μgwell) together with increasing amounts of SGK-1 expression constructs (pCMV6-5XL-hSGK1) at 0.25, 0.5, 1.0 μg/well. An empty vector (pCMV6-5XL) was used for the control transfection. Cells were serum-starved for 24 h and then treated with R1881 (1.0 nM) or the solvent in 5% cFBS-containing media. Luciferase activities were assessed 24 h later and presented as described earlier. Expression levels of SGK-1 or AR were assessed by immunoblotting, and anti-Actin blot was used as loading control. (**c**) LAPC-4 cells were transfected with SGK-1 siRNA or the negative control siRNA for 3 days and then transfected again with the SEAP reporter constructs as indicated. After 24 h serum starvation, cells were stimulated with R1881 or the solvent in 5% cFBS-containing media. SEAP activities were assessed 24 h later as described earlier. Data were from three separated experiments and error bar represents S.E. The asterisk indicates a significant difference ($P<0.05$, *t*-test) compared to the solvent or the vector controls

Figure 6a and b, SGK-1 overexpression significantly enhanced R1881-stimulated ARE-LUC activity, in an SGK-1 dose-dependent manner. In addition, we also noticed that the basal reporter activities in the absence of R1881 were increased by 2–3 folds in SGK-1-transfected cells, although the absolute luciferase reading was much lower than that in R1881-treated cells. These data suggest that SGK-1 overexpression enhances AR transactivation in both the presence or absence of androgen.

Finally, we tested if SGK-1 is required for AR transactivation. We used two SEAP reporters driven by different promoters derived from the endogenous AR target genes, human *psa* (PSA-E2/P-SEAP, termed as PSA-SEAP) and rat *probasin* (ARR_2PB-SEAP, termed as PB-SEAP), as described previously.[33,34] LAPC-4 cells were transfected with SGK-1 siRNA for 3 days to eliminate SGK-1 expression. Cells were then transfected again with the reporter constructs. After R1881 treatment for 24 h, cell culture medium was collected for SEAP activity. As shown in Figure 6c, compared to the solvent control, R1881 stimulation significantly increased SEAP activities from both reporters in cells transfected with the control siRNA. In SGK-1 siRNA-transfected cells, however, R1881-stimulated SEAP activities were completely blocked. Furthermore, the basal SEAP activity (the solvent control) was also slightly reduced in SGK-1 siRNA-transfected cells compared to the control siRNA-transfected cells. These data suggest a critical role of SGK-1 in androgen-stimulated AR transactivation.

Discussion

It is well known that the AR is essential for cellular survival in prostate cancer cells,[8–15] but its downstream effectors are not fully listed. In this study, we sought to identify novel downstream mediators responsible for AR-dependent survival. First, we used a genome-wide approach to screen the genes altered after androgen stimulation. SGK-1 was identified as one of the significantly upregulated genes (Table 1), which was supported by a recent report.[21] AR-dependent SGK-1 expression was then validated at both mRNA and protein levels (Figure 1). After androgen stimulation, the AR was recruited onto the SGK-1 promoter that contains at least two putative motifs for AR binding, which confirms a previous computer-based promoter inspection.[20] Consistently, androgen activated SGK-1 promoter in an AR-dependent manner (Figures 2 and 3). SGK-1 siRNA reduced androgen-mediated death protection from serum starvation (Figures 4 and 5). SGK-1 overexpression not only abrogated AR siRNA-induced apoptosis, but also enhanced AR transactivation in prostate cancer cells (Figure 6); this later event reflects a positive feedback loop of AR-mediated SGK-1 expression and survival, as illustrated in Figure 7. All together, our data demonstrated that SGK-1 is a pivotal factor in AR-dependent survival pathway.

As an early response gene, SGK-1 expression is increased by many external or internal stimuli, including steroid hormones, peptide growth factors, cytokines, and cellular physical or genetic stresses (reviewed by Tessier and Woodgett[18]). We and others have previously identified a GRE motif (5′-CGGACAaaaTGTTCT-3′) at −1159/−1145

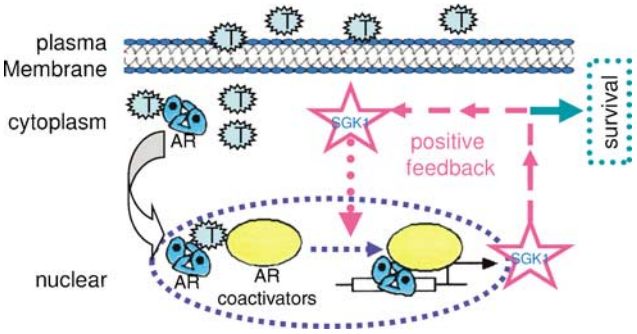

Figure 7 Proposed mechanism for SGK-1 expression in AR-dependent survival. Following androgen stimulation, the androgen receptor (AR) transactivates SGK-1 expression. AR-mediated SGK-1 expression promotes survival and then in turn enhances AR-mediated gene expression, which reflects a positive feedback in AR-dependent survival and gene expression

region in *sgk-1* gene promoter, which is responsible for glucocorticoid-induced SGK-1 expression.[16,24] In this study, we found that the AR also interacted with this region after androgen stimulation. Reporter gene assay showed that the promoter fragment containing this GRE motif was activated by androgen treatment at AR-dependent manner in LNCaP cells that are null of glucocorticoid receptor (GR).[35] Because the AR and the GR are closely related members in the steroid hormone receptor family,[22] it is plausible that this GRE (termed as ARE-1) motif is involved in androgen-induced SGK-1 expression.

In addition to the previously identified GRE/ARE-1 motif, a computer-based motif inspection proposed a secondary GRE/ARE motif in the distal region of −2.5 to −3.2 kb in the *sgk* promoter.[19,20] In this study, our data convincingly indicate that a functional ARE motif indeed exists within this distal region. The promoter fragment (442-SEAP) derived from this distal region functionally responded to androgen stimulation (Figure 2d and e) and the AR was recruited onto this region (Figure 3). A close examination of the promoter sequence revealed a putative ARE motif (5′-GGCTATcccTGTTCT-3′, termed as ARE-2) at the region of −2965 to −2950 related to the transcription site. Although the actual identity of the secondary ARE motif is under further investigation, our data indicate that this distal region containing the ARE-2 motif might act as an enhancer in androgen-induced SGK-1 expression.

Because of its close relationship with Akt, SGK-1 was considered as a major cellular survival factor. A previous report demonstrated that SGK-1, but not Akt, is induced by various environmental stress stimuli and then subsequently protects cell from apoptosis induced by environmental stress.[27] Similarly, in this study, we determined that SGK-1 but not other *sgk* isoforms or Akt was upregulated after androgen stimulation. Our data also demonstrated that SGK-1 is critical for androgen-mediated survival in prostate cancer cells because eliminating SGK-1 expression abrogated androgen-induced death protection.

Early studies demonstrated that SGK-1 exerts its survival function via inactivating proapoptotic transcription factor FKHRL1.[26,27,29,36] A recent study discovered a novel mechanism that SGK-1 promotes NF-κB survival pathway by activating IKKβ.[28] In this study, we found that in AR

siRNA-induced apoptosis, IκB-α phosphorylation was largely reduced in association with SGK-1 downregulation (Figure 5d). Conversely, SGK-1 overexpression reduced AR siRNA-induced apoptosis, which was associated with increased IκB-α phosphorylation. These data indicate that SGK-1 downregulation due to AR silencing resulted in reduction of IKKβ activity, leading to loss of IκB-α phosphorylation and eventually apoptotic cell death.

PI3K is a major factor involved in multiple cellular processes; both Akt and SGK-1 are PI3K downstream targets.[18] We previously showed that PI3K activity is required for androgen-induced AR transactivation and gene expression, and that Akt is dispensable for AR transactivation.[33] In this study, we found that SGK-1 is required for AR-dependent cellular survival and gene expression, indicating that SGK-1 might be one of the PI3K downstream effectors in androgen signaling. Although both Akt and SGK-1 use a similar phosphorylation preference of RxRxxS/T on their substrates, some distinctions were recently reported in terms of their substrate specificity requirements.[37] This substrate specificity might be the reason for the requirement of SGK-1 but not Akt in AR signaling, which will be worthwhile for further investigation.

In conclusion, we demonstrated in this study that protein kinase SGK-1 is an androgen-inducible gene, and is required for AR-dependent cellular survival and gene expression in prostate cancer cells. Further study is desirable to understand the mechanisms for SGK-1 regulation of AR signaling.

Materials and Methods

Cell culture and reagents. The human prostate cancer LNCaP and LAPC-4 cells and their culture conditions were described previously.[12,33,38–40] LAPC-4/SGK1 and LNCaP/SGK1 sublines were established by stably infecting the parental cells with a retrovirus vector (pMSCV-puro-SGK1)[29] bearing the rat *sgk1* gene. The control subline LAPC-4/Puro and LNCaP/Puro were established when an empty vector was used. The stable clones were selected in puromycin-containing media. A mammalian expression vector harboring human *sgk1* cDNA (pCMV6-XL5-hSGK1, OriGene Technologies Inc., Rockville, MD) was used for transient transfection. Antibodies against human AR, SGK-1, Akt, β-Actin as well as secondary antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). Antibodies for human caspase-3, PARP, phospho-specific IκB-α, and plain IκB-α were obtained from Cell Signaling (Beverly, MA). cFBS and regular FBS were obtained from Atlanta Biologicals (Norcross, GA). The synthetic androgen R1881 was purchased from Perkin-Elmer (Wellesley, MA). Other reagents were supplied by Sigma (Saint Louis, MO).

RNA extraction, RT-PCR, real-time RT-PCR, and cDNA microarray. Total RNA was prepared using TriZol™ reagent (Invitrogen Co., Carlsbad, CA). To assess mRNA expression, RT-PCR was carried out using a RETROscript™ kit from Ambion Inc. as per the manufacturer's manual (Austin, TX). The primers and PCR conditions were described as follow: for human *sgk1* gene (forward 5′-atgacggtgaaaactgaggc-3′; backward 5′-gaggtgtcttgcggaatttg-3′); for human *akt1* gene (forward 5′-gtgaaggagggctggctgcacaaacgag-3′; backward 5′-gggtgagcctgatcggaagtccatc-3′). For human PSA gene and 28S ribozyme RNA, the primer sequences were described in our previous publication.[12] The primers were synthesized by IDT Inc. (Coralville, IA). The amplification profile was as follows: 95°C for 30 s, 56°C for 30 s, and 72C for 1 min running in a total of 25 cycles. After 25 amplification cycles, the expected PCR products were size fractionated onto a 2% agarose gel and stained with ethidium bromide.

The real-time PCR was performed using the Applied Biosystems 7500 system and the TaqMan™ Gene Expression Assay kits (Applied Biosystems, Foster City, CA). Reaction mixture consisted of diluted cDNA sample solution (1.0 μl for both SGK-1 and β-actin), 10 μl of TaqMan™ universal PCR master mixture, 1.0 μl of FAM conjugated primer mixtures (Catalog# 4331182). Final volume was 20 μl with distilled water. Denaturizing at 95°C for 15 s, and annealing and extension at 60°C for 1 min was then performed. The denaturizing/annealing cycle was repeated 40 cycles. Relative value of *sgk1* gene expression compared to the control was normalized against β-actin in each sample.

LNCaP cells were used for cDNA-based microarray analysis of androgen-stimulated gene expression. After serum starvation for 48 h, cells were treated with R1881 (1.0 nM) in serum-free media whereas the control cells received the solvent in the same media. At the end of treatment, total cellular RNAs were extracted using a Trizol-based protocol as described earlier. Genechip® arrays (Affymetrix Inc., Santa Clara, CA) was utilized and the protocol was described in our recent publication.[38] Briefly, 10 μg of total RNAs were used for the first-strand cDNA synthesis with the SuperscriptChoice system (Invitrogen). The *in vitro*-transcription labeling was conducted using the EnzoBio array high-yield RNA transcript labeling kit (Affymetrix). Genechips were hybridized for 16 h at 45°C and 60 r.p.m. in a Genechip hybridization oven. After washing, the signal intensity was detected using an Agilent Gene Array scanner. Microarray data were analyzed using GeneSpring software (Agilent Technologies, Inc.).

Western blot analysis. For protein expression analysis, western blot was performed as described previously.[12,33,38–40] Briefly, after treatment, cells were pelleted and lysed in a buffer containing protease inhibitors (Half™ Protease Inhibitor Cocktail Kit, PIERCE, Rockford, IL), separated on SDS-PAGE gels and transferred to PVDF membrane (BIO-RAD, Hercules, CA). Membranes were blocked in a Tris-buffered solution plus 0.1% Tween-20 (TBS-T) solution with 5% nonfat dry milk and incubated with primary antibodies overnight at 4°C. Immunoreactive signals were detected by horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotech, Santa Cruz, CA) followed by SuperSingnal® WestFemto Maximum Sensitivity Substrate (PIERCE Biotechnology, Rockford, IL).

ChIP analysis. The ChIP assay was performed using a ChIP-IT™ assay kit (Active Motif, Carlsbad, CA) as described in our recent publication.[38] Cells were maintained in 15-cm dishes and serum-starved for 24 h. After pretreatment with anti-androgen Bicalutamide (Casodex, CSDX), cells were stimulated with R1881. Polyclonal antibodies against AR (Clone C815), RNA polymerase II, GAPDH, and normal rabbit serum were obtained from Santa Cruz Biotech (Santa Cruze, CA). Proper control experiments were performed as described in our recent publication.[38] The primers for the PCR reactions were: fragment −3382/−2940 (442a 5′-gataagtttagccgaccatccg tctc-3′; 442b 5′-gagccagagacggctatccctgttc-3′); fragment −1548/−1130 (418a 5′-caccggcatcgctg ttctgcagag-3′; 418b 5′-ctgagaac attttgtccgttccgc-3′); fragment −852/−290 (562a 5′-ctgagtcacggcg gctccttcaag-3′; 562b 5′-ctctctcatttattccgccgccggag-3′). The primer binding sites were illustrated in Figure 2a.The PCR products were separated on 1% agarose gels and stained with ethidium bromide for visualization.

Reporter plasmid construction and reporter gene assays. The pGL3-SGK1/F4R4-Luc reporter construct was described previously,[24] and the internal control reporter pCMV-SEAP, expressing the secreted alkaline phosphatase (SEAP) under the control of the cytomegalovirus (CMV) promoter and the reporter assays were performed as described previously.[12,33,38,40] To generate different promoter fragments (Figure 2a) from *sgk1* promoter, the PCR fragments amplified from LNCaP cell genomic DNA using the primers described in ChIP assays were subcloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) and the accuracy of the inserts were confirmed by direct sequencing. Then, the individual fragment was released from the pCR2.1 vector using *Hind*III/*Xba*I digestion and directionally ligated into pSH1-ARR2PB-SEAP vector as described previously,[33] so as to generate *sgk1* fragment-driven SEAP gene reporters by replacing the probasin promoter. The ARE-LUC reporter was described previously.[41]

The reporter gene assay was performed as described previously.[12,33,38,40] Briefly, cells plated in six-well tissue culture plates were transfected with reporter constructs using the Cytofectene™ reagent (BioRad) according to the manufacturer's protocol. After 24 h, cells were serum-starved for another 24 h, and then pretreated with anti-androgen CSDX or the solvent. Cell were treated with the solvent or stimulated with R1881 in 5% cFBS. Culture supernatants and cell lysates were collected for reporter assays. The luciferase reporter activity of each sample was normalized against the corresponding SEAP activity and protein concentration prior to calculation of the relative fold induction compared to the controls.

SiRNA transfection, cell viability, and clonogenic assay. Predefined siRNA against human *sgk1* gene and a negative control siRNA with scramble sequence were purchased from Santa Cruz Biotechnology and transfected with OligoFectamine™ (Invitrogen) according to the manufacturer's manual. For cell viability experiments, cells were seeded at the density of 2×10^4 cells in 12-well plates in duplicates and allowed to attachment overnight. One week after siRNA transfection, cells were subjected to viability assay. Cells were harvested at the end of experiments and the number of viable cells was counted using a hemocytometer after staining with trypan blue as described previously.[12,40]

For the clonogenic assay, 1.0×10^3 cells were seeded in a 35-mm dish and transfected with SGK-1 siRNA or the control siRNA. The cultures were monitored daily for colony formation in a 2% cFBS-containing media. One week later, the cultures were washed with phosphate-buffered saline (PBS), fixed, and stained as described previously.[40] The colonies were counted and then photographed using a digital camera.

AAV production and infection. To silence AR gene expression, a hairpin-structured expression vector was created using pSilencer-U6 1.0 plasmid vector (Ambion Inc., Austin, TX) based on our previously published AR siRNA sequence.[12] The oligonucleotide sequences were as follow: sense, 5′-GAAGGCCAGTTG TATGGAC*ttcaagaga*GTCCATACAA CTGGCCTTC ttttt-3′ and antisense, 5′-attaa aaaaGAAGGC CAGTTGTATGGAC*tctcttgaa*GTCCATACAACT GGCCTTC*ggcc*-3′. Annealed DNA fragment was subcloned into *Apa*I/*Eco*RI sites of pSilencer-U6 vector, and the resultant vector named as pSilencer-U6-ARHP8. To create an AAV for ARHP8 siRNA expression, the AAV Helper-free system (Stratagene, La Jolla, CA) was utilized. The ARHP8 expression cassette was released by *Xba*I digestion and subcloned into pCMV-MCS from the AAV system. Then, this fragment was moved into pAAV-IRES-hGFP by *EcoR*I/*Xho*I digestion/ligation process to generate AAV-ARHP8 viral-producing vector. The pAAV-IRES-hGFP construct was used to produce an empty AAV virus (AAV-GFP). These AAV particles were packaged and purified by Applied Viromics LLC (Fremont, CA) at a titer of 10^{12} viral particle per ml.

Statistical analysis. All experiments were repeated two or three times. RT-PCR results are presented from a representative experiment. The mean and standard error (S.E.) from multiple experiments for real-time RT-PCR, cell viability, or reporter gene assay are shown. The significant differences between groups were analyzed as described previously[12,33,38–40] using the SPSS computer software (SPSS Inc., Chicago, IL).

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Androgen Receptor-Dependent Regulation of Bcl-xL Expression: Implication in Prostate Cancer Progression

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BACKGROUND. Recently we reported that silencing the androgen receptor (AR) gene reduced Bcl-xL expression that was associated with a profound apoptotic cell death in prostate cancer cells. In this study we further investigated AR-regulated Bcl-xL expression.

METHODS. Prostate cancer cell line LNCaP and its sublines, LNCaP/PURO and LNCaP/Bclxl, were used for cell proliferation assay and xenograft experiments in nude mice. Luciferase gene reporters driven by mouse or human *bcl-x* gene promoter were used to determine androgen regulation of Bcl-xL expression. RT-PCR and Western blot assays were conducted to assess Bcl-xL gene expression. Chromatin immunoprecipitation assay was performed to determine AR interaction with Bcl-xL promoter. Bcl-xL-induced alteration of gene expression was examined using cDNA microarray assay.

RESULTS. In cultured prostate cancer LNCaP cells, androgen treatment significantly increased Bcl-xL expression at mRNA and protein levels via an AR-dependent mechanism. Promoter analyses demonstrated that the AR mediated androgen-stimulated *bcl-x* promoter activation and that the AR interacted with *bcl-x* promoter. Enforced expression of Bcl-xL gene dramatically increased cell proliferation in vitro and promoted xenograft tumor growth in vivo. Genome-wide gene profiling analysis revealed that Bcl-xL expression was significantly higher in metastatic and castration-resistant diseases compared to normal prostate tissues or primary cancers. Bcl-xL overexpression significantly increased the expression of cyclin D2, which might be responsible for Bcl-xL-induced cell proliferation and tumor growth.

Abbreviations: AR, androgen receptor; CDK, cyclin-dependent kinase; FBS, fetal bovine serum; NF-κB, nuclear factor kappa B; PCR, polymerase chain reaction; PURO, puromycin; RT, reverse transcription; SE, standard error; START, signal transducer and activator of transcription; TBS-T, Tris-buffered solution plus Tween 20.

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CONCLUSIONS. Taken together, our data strongly suggest that androgen stimulates Bcl-xL expression via the AR and that increased Bcl-xL expression plays a versatile role in castration-resistant progression of prostate cancer. *Prostate 9999: 1–9, 2007.*

KEY WORDS: **prostate cancer; androgen receptor; bcl-xl; cell proliferation; xenograft**

INTRODUCTION

It has been widely accepted that prostate growth and differentiation is androgen-dependent, and the androgen receptor (AR) plays a critical role in the development and progression of prostate cancer [1]. Androgen withdrawal triggers apoptosis in both normal and malignant prostate epithelial cells but castration-resistant prostate cancer cells do not undergo apoptosis, suggesting that AR-mediated survival signal is reactivated or prostate cancer cells may utilize alternative cellular pathways for their survival (reviewed in Ref. [1,2]). So far, little is known about the downstream effecters of AR-mediated survival pathway.

The *bcl-x* gene encodes multiple spliced mRNAs, of which Bcl-xL is the major transcript [3,4]. Like Bcl-2, Bcl-xL protects cells from apoptosis by regulating mitochondria membrane potential and volume, by interacting with pro-apoptotic members Bax or Bim, and subsequently prevents the release of cytochrome *c* and other mitochondrial factors from the inter-membrane space into cytosol. In addition, Bcl-xL may prevent apoptosis via a cytochrome *c*-independent pathway [5]. Although Bcl-xL protein can be regulated post-transcriptionally, it is mainly controlled at the gene expression level (reviewed in Ref. [6]). The *bcl-x* promoter contains consensus motifs for a number of transcription factors, including Sp1, AP-1, Oct-1, Ets, Rel/NF-κB, STATs (signal transducer and activator of transcription), and GATA-1, in which three transcription factor families, STATs, Rel/NF-κB, and Ets family, have been demonstrated to play an important role in the regulation of *bcl-x* gene expression. In addition, steroid hormone receptors including receptors for glucocorticoids and progesterone have been reported to bind to the mouse *bcl-x* promoter [7,8].

In an earlier report, Bcl-xL protein was detected in the epithelial cells of normal prostate gland and prostate cancers [9]. Later on, increasing levels of Bcl-xL expression were found to correlate with higher grade/stage in prostate cancers, indicating a possible role of Bcl-xL in prostate cancer progression [10,11]. Blocking Bcl-xL expression in prostate cancer cells decreased cell proliferation [12–14]. Most interestingly, we recently found that silencing the *AR* gene in prostate cancer cells significantly reduced Bcl-xL expression, which was associated with a profound apoptotic cell death, suggesting that Bcl-xL is a downstream effecter for AR-dependent survival in prostate cancer cells [15]. Therefore, in this study, we went on to investigate AR-dependent regulation of Bcl-xL expression. Our data indicated that the AR regulates Bcl-xL expression at the transcription level, and that Bcl-xL overexpression promotes castration-resistant progression of prostate cancer.

MATERIALS AND METHODS

Cell Culture, Reagents and Antibodies

Human prostate cancer LNCaP and the stable sublines (LNCaP/Bclxl and LNCaP/PURO) were described previously [15]. Briefly, LNCaP/Bclxl cells were established by a co-transfection of LNCaP cells with a vector bearing HA-tagged human *bcl-xl* cDNA sequence (pcDNA3.1-Bclxl.HA) and a vector harboring the puromycin resistant gene (pBABE-PURO). LNCaP/PURO cells were established by transfection of pBABE-PURO as a control. Cell lines were maintained in a humidified atmosphere of 5% CO_2, RPMI 1640 supplemented with 10% fetal bovine serum (FBS) with antibiotics (Invitrogen, Carlsbad, CA). Antibodies for cyclins, Bcl-2, Bax, Bcl-xL and Actin were purchased from Santa Cruz Biotech (Santa Cruz, CA). Polyclonal anti-AR antibody, normal rabbit serum and insulin-like growth factor-1 (IGF-1) were obtained from Sigma (St. Louis, MO). Androgen antagonist bicalutamide was a generous gift from AstraZeneca. Charcoal-stripped FBS (cFBS) was purchased from Atlanta Biologicals (Norcross, GA). PCR primers were synthesized by IDT (Coralville, IA).

Cell Proliferation Analysis

Cells were seeded at a density of 2×10^4 cells/well in 12-well plates in triplicates and allowed to attachment overnight. Cells were then maintained in various conditions as indicated in the figures. The viable cells was counted using a hemocytometer after staining with trypan blue as described in our recent publication [15,16].

Affymetrix cDNA-Based Microarray and ReverseTranscription-PCR

Cells exponentially grown in 10% FBS-containing media were harvested and total cellular RNAs were

extracted using a Trizol-based protocol as described [15,16]. Genechip® arrays (Affymetrix Inc., Santa Clara, CA) were used to determine gene expression profiles as described [16]. Briefly, transcription labeling was conducted using the EnzoBio array high yield RNA transcript labeling kit (Affymetrix Inc.). The first strand cDNA was synthesized using the SuperscriptChoice system (Invitrogen). Genechips were hybridized for 16 hr at 45°C and 60 rpm in a Genechip hybridization oven Model 640. After washing, the signal intensity was detected using an Agilent Gene Array scanner.

Reverse transcription (RT)-PCR was conducted to assess gene expression at the mRNA level. Total cellular RNAs from the cDNA microarray experiments were used for RT-PCR as described [15,16]. The PCR primer sequences for the target genes were listed in Table I. The GAPDH gene was set as the internal control as described in our previous study [17]. The following PCR conditions were used: 95°C for 45 sec; 56°C for 30 sec; 72°C for 60 sec at 25 cycles. The PCR products were separated in a 2% agarose gel and visualized with ethidium bromide under UV light [15–17].

Western Blot Analysis

Western blot was carried out as described previously [15–17]. Briefly, cells were pelletted and lysed in a buffer containing protease inhibitors (Half™ Protease Inhibitor Cocktail Kit, PIERCE, Rockford, IL). Equal amounts of proteins were separated on SDS–PAGE gels and transferred to PVDF membrane (Bio-Rad, Hercules, CA). Membranes were blocked in a Tris-buffered solution plus 0.1% Tween 20 (TBS-T) solution with 5% nonfat dry milk and incubated with primary antibodies overnight at 4°C. Immunoreactive signals were detected by horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotech) followed by SuperSingnal® WestFemto Maximum Sensitivity Substrate (PIERCE).

Luciferase Gene Reporter Assay

The pGL2-3.2Luc reporter construct contains the firefly luciferase gene controlled by a 3.2 kb fragment from the promoter region of mouse *bcl-x* gene [18]. The other luciferase reporter driven by a human *bcl-x* promoter (pXP-BclxL^{Pr}-LUC) was described previously [19]. The internal control reporter pCMV-SEAP expresses the secreted alkaline phosphatase (SEAP) under the control of the cytomegalovirus (CMV) promoter. The reporter assays were performed as described previously [16,17]. Briefly, cells plated in 6-well tissue culture plates were transfected with reporter constructs using the Cytofectene™ reagent (BioRad) according to the manufacturer's protocol. After 24 hr, cells were serum-starved for another 24 hr, and then treated with R1881, IGF-1 or the solvent overnight. Culture supernatants and cell lysates were collected for reporter assays. The luciferase reporter activity of each sample was normalized against the corresponding SEAP activity and protein content prior to calculation of the fold induction value relative to the control.

Chromatin Immunoprecipitation (ChIP) Analysis

Cells were maintained in 10-cm dishes and serum-starved for 24 hr before treatment. Bicalutamide were added for 45 min followed by R1881addition in 2% cFBS for 12 hr. The ChIP assay was performed using a ChIP assay kit (Upstate, Charlottesville, VA), and several control experiments were conducted as described in our recent publication [16,20]. Primers for the PCR were 5′-cgatggaggaggaagcaagc-3′ and 5′-gcaccacctacattcaaatcc-3′, which amplify a 250-bp fragment corresponding to human *bcl-x* gene promoter sequence −384 to −634 from the TATA Box site (GenBank™ accession number D30746). The PCR products were separated on 1% agarose gels and stained with ethidium bromide for visualization.

Mouse Xenograft Experiments

Athymic male mice (Charles River, Wilmington, MA) were maintained in accordance with the Institutional Animal Care and Use Committee procedures and guidelines. LNCaP/PURO and LNCaP/Bclxl cells were treated with trypsin, resuspended in PBS, and injected

TABLE I. PCR Primer Sequences

Gene name	Sequences (forward/backward)
CCNA2	TTATTGCTGGAGCTGCCTTT/ACTGTTGTGCATGCTGTGGT
CCNE2	CAGGTTTGGAGTGGGACAGT/CTTCCTCCAGCATAGCCAAA
CCND1	CCTGTCCTACTACCGCCTCA/CAGTCCGGGTCACACTTG
CCND2	CTGGGGAAGTTGAAGTGGAA/CTTAAAGTCGGTGGCACAC
CCND3	TGGATGCTGGAGGTATGTGA/GCACAGTTTTTCGATGGTCA
Bcl2L1(bcl-xl)	GGCTGGGATACTTTTGTGGA/GAAGAGTGAGCCCAGCAGAA
GAPDH	ACCACAGTCCATGCCATCAC/TCCACCACCCTGTTGCTGTA

subcutaneously into the rear flanks (2×10^6 cells/flank) of 6-week-old intact or castrated mice as described in our recent publication [21]. Tumor growth was monitored for 5 weeks. The tumor volumes were determined by measuring the length (L) and the width (W) and calculating the volume ($V = L \times W^2/2$), as described [21].

Statistical Analysis

All cell culture-based experiments were repeated two or three times. RT-PCR and Western blot are presented from representative experiments. The mean and SEM for luciferase assay, cell proliferation and tumor growth are shown. The significant differences between groups were analyzed as described previously [15–17] using the SPSS computer software (SPSS Inc., Chicago, IL).

RESULTS

AR-Dependent Regulation of Bcl-xL Expression

We recently found that silencing the AR gene in prostate cancer cells led to significant reduction of Bcl-xL expression [15], suggesting that Bcl-xL expression is positively modulated by androgens/AR pathway. To test this hypothesis, human prostate cancer LNCaP cells were stimulated with a synthetic androgen R1881 and Bcl-xL expression was determined at the mRNA level by RT-PCR. As shown in Figure 1A, R1881 increased Bcl-xL expression in a time-dependent manner. Next, we determined if the AR is required for androgen-stimulated Bcl-xL expression. LNCaP cells were treated with R1881 in the presence or absence of AR antagonist bicalutamide. As shown in Figure 2B, R1881 strongly increased Bcl-xL protein levels, which was blocked by bicalutamide pretreatment, indicating that the AR is required for androgen-stimulated Bcl-xL expression.

Next, we conducted a luciferase reporter gene assay with a 3.2 kb mouse *bcl-x* gene promoter [4] to determine if androgen stimulates Bcl-xL expression by activating its promoter. As shown in Figure 1C, R1881 significantly stimulated the mouse *bcl-x* promoter activity in a dose-dependent manner, similar as

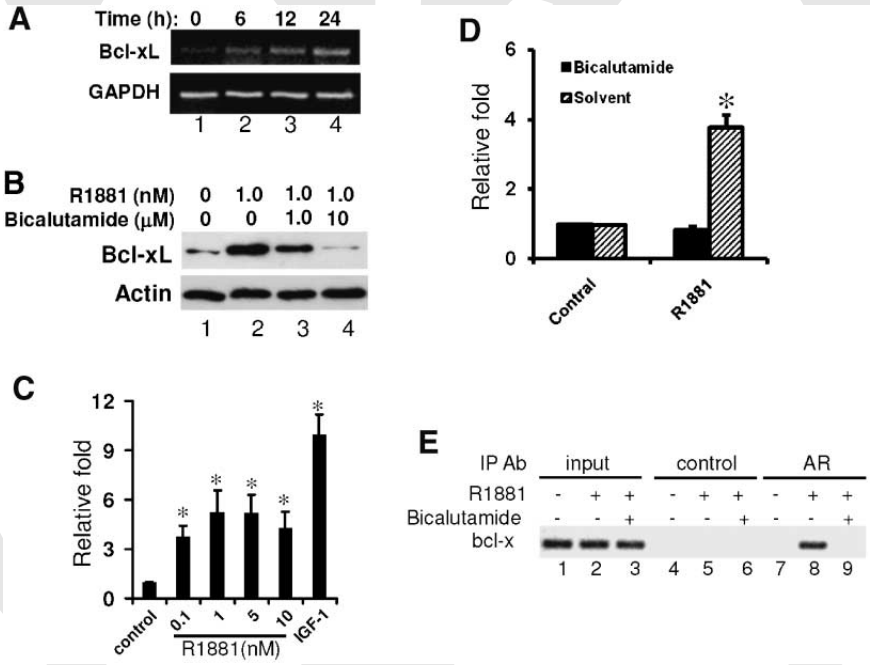

Fig. 1. AR modulates Bcl-xL expression. **A**: LNCaP cells were serum-starved for 24 hr and then treated with R1881 (1.0 nM). Cells were harvested at different time points as indicated and total RNA was extracted for RT-PCR. **B**: LNCaP cells were serum-starved for 24 hr and then treated with R1881 or the solvent after a 45 min pre-treatment with bicalutamide at different doses. Cells were harvested and whole cell lysates were subject for Western blot assay. Actin blot served as loading control. **C**: LNCaP cells were transfected with pGL2-3.2LUC and pCMV-SEAP reporter constructs overnight in serum-free condition and then treated with R1881 at different doses as indicated or IGF-1 (100 ng/ml) in the presence of 2% cFBS. Culture media and cells were harvested for reporter gene assay as described [15–17]. **D**: LNCaP cells were transfected with pXP-BclxL^{pr}-LUC and pCMV-SEAP reporter constructs overnight in serum-free condition and then treated with R1881 (1.0 nM) or the solvent after a 45 min pretreatment of bicalutamide (10 μM). Reporter gene assay was performed as described [15–17]. **E**: LNCaP cells were serum-starved and then treated with R1881 (1.0 nM) after a 45 min pretreatment of bicalutamide (10 mM). Cells were harvested 12 hr later for ChIP assay. Rabbit polyclonal anti-AR antibodies were utilized to immunoprecipitate AR-bound chromatin DNA. Normal rabbit serum was used for the control experiments. INPUT represents 10% of total chromatin DNA. Data were from two (A,B,E) or three (C,D) separate experiments. Error bar indicates SE. The *asterisk* indicates a significant difference between treatments versus the controls (Student's *t*-test, $P < 0.05$).

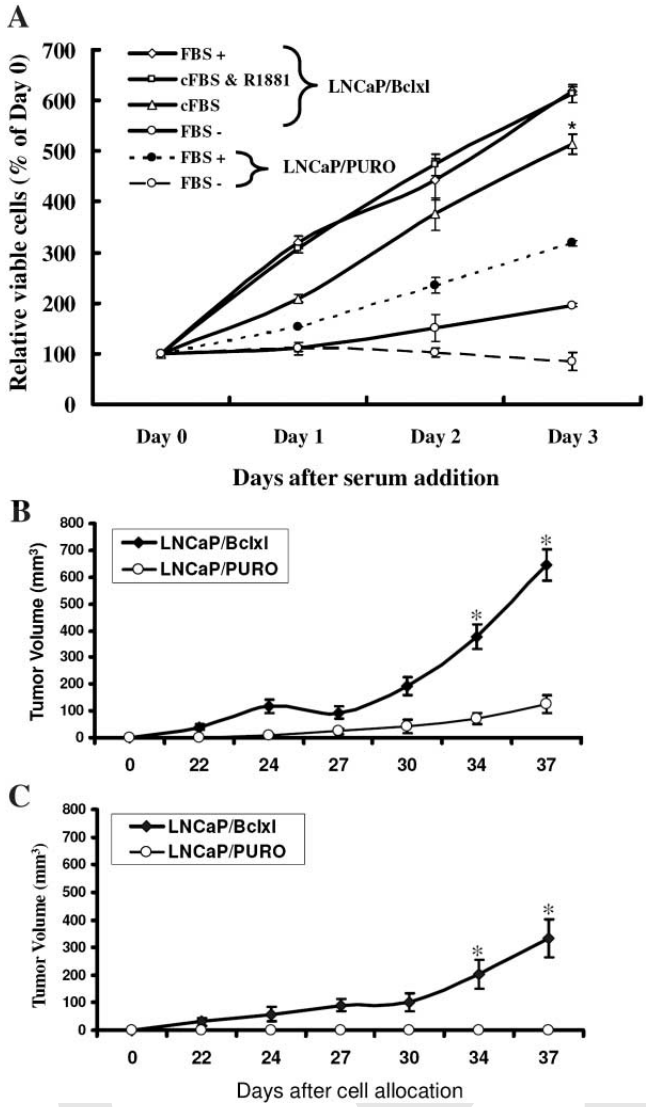

Fig. 2. Bcl-xL overexpression enhances cell proliferation and promotes tumor growth. **A**: LNCaP/PURO and LNCaP/Bclxl cells were seeded in 12-well plates at a density of 2×10^4 cells per well. After attachment, cells were subjected to different culture condition as indicated. Viable cells were counted every day after trypan blue staining. Data presented as a relative percentage of cell numbers over the initial cell density from three different experiments. Error bar indicates SE. The *asterisk* indicates a significant difference compared to LNCaP/PURO cells with FBS+ condition (Student's *t*-test, $P < 0.05$). **B**,**C**: LNCaP/PURO and LNCaP/Bclxl cells (2×10^6) were injected subcutaneously into the rear flank of gonadal intact or castrated nude mice ($n = 5$), as described in our recent publication [21]. Xenograft development and tumor growth were monitored every day for 5 week. Error bar indicates SE, and the *asterisk* indicates a significant different between the two groups.

IGF-1 that is a strong inducer of Bcl-xL expression [22]. We also utilized another luciferase reporter driven by a human *bcl-x* promoter (pXP-Bclx^{Pr}-LUC, 19). As shown in Figure 1D, R1881 significantly increased the promoter activity, which was completely blocked by bicalutamide pretreatment, indicating the involvement of the AR.

Then, we performed a ChIP assay to determine if the AR physically binds to human *bcl-x* promoter. Primer pairs for the PCR reaction were designed to target a region that is located within the promoter fragment used in the above luciferase assay (Fig. 1D). As shown in Figure 1E, R1881 treatment induced the AR binding to the promoter of the *bcl-x* gene, while pretreatment with bicalutamide totally abolished this interaction. After a close examination of the promoter sequence, a putative androgen responsive element (ARE) was noticed −459/−444, 5′-tgTACAgatTGTTtTg-3′. The capital letters indicate the consensus sequences based on our previous description [20]. Further analysis of this putative ARE is being conducted by our group. These data clearly demonstrated that the AR is involved in androgen-induced transcriptional regulation of Bcl-xL expression.

Bcl-xL Overexpression Promotes Cell Proliferation and Androgen-Independent Progression

In addition to its anti-apoptotic effect, Bcl-xL was recently shown to modulate cell cycle progression [23–25]. Blocking Bcl-xL expression in prostate cancer cells largely reduced cell proliferation [12]. Therefore, we determined if Bcl-xL overexpression would promote cell growth. Two LNCaP cell sublines, LNCaP/Bclxl and LNCaP/PURO, established previously [15], were used in the experiments. Cell proliferation curve was compared among different culture conditions for both cell lines. As shown in Figure 2A, in the absent of serum, cell proliferation was almost stopped in both cell lines. Under the condition of 10% FBS, LNCaP/Bclxl cells were growing significantly faster than LNCaP/PURO cells, indicating that Bcl-xL overexpression enhanced cell proliferation. Under cFBS condition (androgens were depleted), LNCaP/Bclxl cells kept growing, although it was slightly slower than that in FBS condition. R1881 restored LNCaP/Bclxl cell proliferation in cFBS media similar to FBS condition. These data indicated that Bcl-xL promotes androgen-independent cell proliferation of prostate cancer cells in vitro.

To determine if Bcl-xL induces castration-resistant phenotype of prostate cancer in vivo, we established xenografts in nude mice using these LNCaP sublines (LNCaP/Bclxl and LNCaP/PURO). Xenograft tumor growth was monitored in gonadal intact or castrated mice. In gonadal intact animals, palpable xenografts were observed in about 3–4 weeks after LNCaP/PURO cell inoculation, while LNCaP/Bclxl xenograft tumors appeared about 1 week earlier than LNCaP/PURO

xenografts (Fig. 2B,C). Furthermore, LNCaP/Bclxl xenograft tumors exhibited a significant higher growth rate than the counterpart LNCaP/PURO tumors (Fig. 2B), indicating that Bcl-xL overexpression promoted tumor growth in vivo. In castrated animals, as expected, LNCaP/PURO cells failed to produce any palpable xenografts (Fig. 2C), reflecting a castration-sensitive feature of LNCaP cells. However, LNCaP/Bclxl cells produced xenograft tumors within 3–4 weeks in castrated animals (Fig. 2C), indicating that Bcl-xL overexpression promoted castration-resistant tumor growth. However, the growth rate of LNCaP/Bclxl xenografts was lower in castrated animals than that in intact animals, suggesting that other factors besides Bcl-xl are required for castration-resistant tumor growth. These in vivo data were consistent with Bcl-xL-induced cell proliferation in vitro (Fig. 2A), and demonstrated that Bcl-xL is a factor involved in castration-resistant tumor growth of prostate cancer in vivo.

Increased Bcl-xL Expression Correlates With Prostate Cancer Progression

Previous studies with immunohistochemical method showed a close correlation of Bcl-xL expression at protein level with disease progression of human prostate cancers [10,11]. To determine Bcl-xL expression at the mRNA level in human prostate cancers, we re-analyzed a published cDNA microarray dataset and the information of patient population and clinical data were described previously [26]. Briefly, human specimens from normal prostate, primary tumors, tumors after androgen ablation therapy, metastatic tumors as well as tumor samples from castration-resistant cases were used in the microarray analysis on the *Affymetrix* HG-U95 chip [26]. As shown in Figure 3, Bcl-xL expression was significantly higher in primary tumors compared to normal tissues. After hormone therapy, Bcl-xL expression dropped dramatically compared to primary tumors. In metastatic/castration-resistant tumors, Bcl-xL expression increased significantly compared to primary tumors and normal tissues. This trend of alteration in Bcl-xl expression was in parallel with the changes of AR expression as described previously [26]. In contrast, another anti-apoptotic protein Bcl-2 expression did not show any obvious differences between normal tissue and primary tumors. However, Bcl-2 levels increased significantly after androgen ablation compared to primary tumors and normal tissues, which was consistent with a previous report [27] that showed an androgen-mediated repression of Bcl-2 expression. The pro-apoptotic member Bax levels remained unchanged among these patient groups. These findings suggest

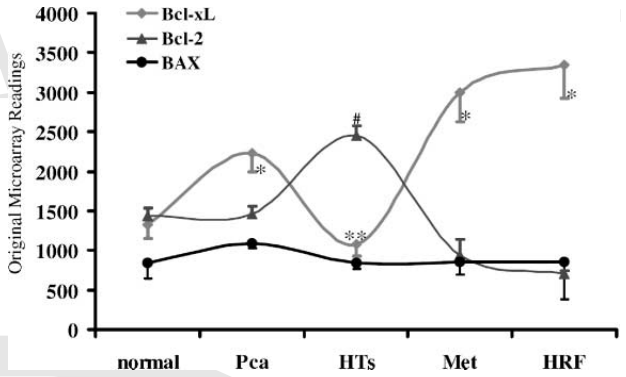

Fig. 3. Bcl-xL expression correlates with prostate cancer progression. Raw data (Mean) from a pre-published cDNA microarray analysis were graphed based on different patient groups, as described [26], including normal prostate samples (n = 5), primary tumors (Pca, n = 23), tumors after hormone therapy (HTs, n = 17), metastatic tumors (Met, n = 9) and castration-resistant tumors (HRF, n = 3). Error bar indicates SE. The single *asterisk* indicates a significant difference compared to "normal" group ($P < 0.01$), the double *asterisk* indicates a significant difference compared to "Pca" group ($P < 0.05$); # indicates a significant difference compared to "normal/Pca" groups ($P < 0.05$). Analysis was performed using Student's *t*-test.

that Bcl-xL expression is associated with the levels of AR expression/activity during prostate cancer progression, especially in castration-resistant stage.

Bcl-xL Overexpression Increases Cyclin D2 But Decreases Cyclin DI Expression

To understand the mechanisms underlying Bcl-xL-induced cell proliferation/tumor growth, we performed a genome-wide screening of gene expression at the mRNA level. Total RNAs used for the analysis were extracted from exponentially grown LNCaP/Bclxl and LNCaP/PURO cells. Because cyclins and their associated cyclin-dependent kinases (CDKs) are the major driving forces for cell proliferation/growth, we focused on cyclin-related genes. As shown in Table II, the most significant changes were cyclin D2 up-regulation and cyclin D1/G2 down-regulation. All CDKs and other cyclins remained unchanged. We then confirmed these alterations of gene expression in RT-PCR experiments. As shown in Figure 4A, increased expression of cyclin D2 and decreased expression of cyclin D1 were observed in LNCaP/Bclxl cells compared to LNCaP/PURO cells, which were consistent to the cDNA microarray data. These alterations of gene expression were further confirmed at protein levels by Western blot analysis (Fig. 4B). These results suggest that increased expression of cyclin D2 is one of the mechanisms responsible for Bcl-xL-induced cell proliferation/tumor growth. The underlying mechanism

TABLE II. Bcl-xL-Related Changes of Gene Expression

Probe set	Gene symbol	Fold change	LNCaP/Bclxl signal	LNCaP/PURO signal
Up-regulated				
200952_s_at	**CCND2**[Q1]	**6.4**	**128**	**5.5**
200951_s_at	**CCND2**	**5.61**	**112.2**	**6.3**
213226_at	CCNA2	2.12	2476.1	1168.4
203418_at	CCNA2	1.43	1601.2	1123.6
213523_at	CCNE1	1.16	637.2	549
214710_s_at	CCNB1	1.08	5130.4	4768.3
201700_at	CCND3	1.02	763.1	746.9
204252_at	CDK2	1.17	1394.4	1191.3
202246_s_at	CDK4	1.64	5695.7	3476.9
204247_s_at	CDK5	1.41	897.2	638
207143_at	CDK6	1.80	113.2	62.8
203967_at	CDC6	1.26	1045.3	829.3
203968_s_at	CDC6	1.41	1248.5	884.2
211297_s_at	CDK7	1.21	909.9	749.3
204831_at	CDK8	1.47	956.8	649
Down-regulated				
208711_s_at	**CCND1**	**−3.18**	**148.5**	**471.5**
207712_at	**CCND1**	**−2.99**	**361.1**	**1080.1**
211559_s_at	**CCNG2**	**−4.83**	**237**	**1143.6**
202769_at	**CCNG2**	**−3.1**	**570.3**	**1770.3**
202770_s_at	**CCNG2**	**−3.65**	**309.1**	**1128.7**

for Bcl-xl-induced cyclin D2 expression is being conducted by our group.

DISCUSSION

Castration-resistant progression is the major obstacle for curing prostate cancers. To become Castration-resistant, prostate cancer cells need to overcome two major roadblocks, survival and proliferation after castration. It has been demonstrated that the AR is the essential determinant in prostate cancer progression [1,2]. We recently showed that knocking down AR expression led to a profound apoptotic cell death that was concomitant with down-regulation of Bcl-xL expression [15]. In this study, we demonstrated that Bcl-xL expression is modulated by the AR, and that Bcl-xL overexpression increases cell proliferation in vitro and promotes castration-resistant tumor growth in vivo.

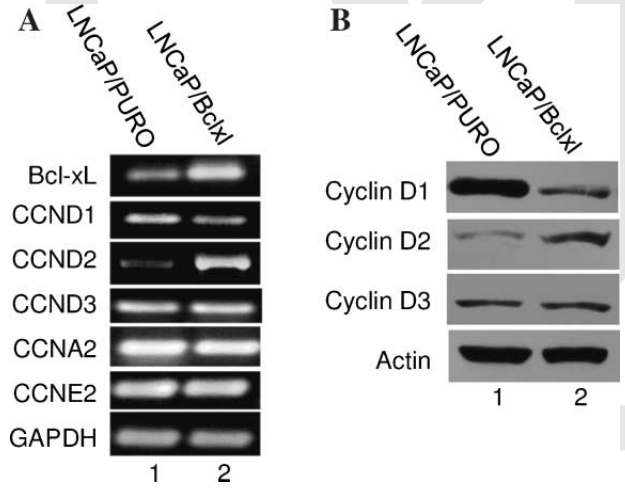

Fig. 4. Bcl-xL overexpression increases cyclin D2 expression. **A**: Total cellular RNA was extracted from exponentially grown cells and was used for RT-PCR reaction. Primer sequences were listed in Table I. **B**: LNCaP/PURO and LNCaP/Bcl-xl cells were harvested and whole cell lysates were extracted. Equal amount of proteins were subjected for Western blot. Actin was used for loading control.

As a transcription factor, the AR regulates a large group of genes that modulate the development, growth and tumorigenesis of the prostate ([28], reviewed in Ref. [29]). In prostate cancer patients, AR-mediated gene expression is largely suppressed after androgen ablation therapy. However, the AR regains its activity once cancer cells relapse in castration-resistant stage ([26], reviewed in Ref. [2]). In this study, we found that Bcl-xL expression was significantly higher in primary cancers compared to the normal control, but it decreased after androgen ablation therapy. However, Bcl-xL level rebound even higher in metastatic and rebound cancers compared to primary cancers and normal control. These patterns of alteration, similar as the AR expression/activation, suggest that Bcl-xL expression is modulated by the AR-related pathway. Indeed, we found that the AR regulates Bcl-xL

expression at the transcriptional level. Our findings are supported by two previous studies that Bcl-xL expression is increased by the glucocorticoid receptor, a close relative of the AR [30,31].

Bcl-xL is one of the major anti-apoptotic proteins in Bcl-2 family [32]. Meanwhile, the involvement of Bcl-2/Bcl-xL in cell cycle control has been reported [23–25]. Adenovirus-mediated delivery of *Bcl-xL* gene enhanced axon regeneration of adult neurons independent of neuronal survival in rat [33]. In cooperation with c-Myc, enforced expression of Bcl-xl gene in B-lymphoid cells caused lympho-proliferative disease and plasma cell malignancies in a transgenic mouse model [34]. In this study, we found that Bcl-xL overexpression dramatically increased cell proliferation of prostate cancer cells in vitro and xenograft tumor growth in vivo. Our results are in agreement with a recent report that blocking Bcl-xL expression reduced cell proliferation in prostate cancer cells [12]. Our further analysis with a comprehensive approach revealed that Bcl-xL-induced cell proliferation might be due to up-regulation of cyclin D2 expression.

D-type cyclins are the G1 phase cyclins, responsible for G1-S transition [35]. Although alternative mechanisms may exist to allow cell cycle progression in a cyclin D-independent fashion [36], each individual cyclin D is capable of driving cell progression through G1 phase to S phase [37]. Overexpression of cyclin D1 has been found in multiple human cancers including prostate cancers [38]. Like cyclin D1, cyclin D2 is also capable to promote hypertrophic growth [39]. However, cyclin D2 was inactivated in some prostate cancer tissues due to aberrant promoter methylation [40]. In this study, we found that cyclin D2 expression level was very low both at the mRNA and protein levels in LNCaP/PURO cells (Fig. 4). Interestingly, cyclin D2 expression increased by more than fivefold in LNCaP/Bcl-xL cells compared to LNCaP/PURO cells. Meanwhile, cyclin D1 expression decreased for two to threefolds and cyclin D3 remained unchanged. These alterations suggest that Bcl-xL shift the expression pattern of D-type cyclins, which may be responsible for the castration-resistant transformation of LNCaP tumors. The mechanism underlying Bcl-xL-induced changes of D-type cyclin expression is under further investigation by our group.

In conclusion, we demonstrated that the anti-apoptotic protein Bcl-xL is an AR-regulated gene, and its expression levels in prostate cancers correlates with disease progression. Enforced expression of Bcl-xL protein increases cell proliferation in vitro and promotes xenograft tumor growth in vivo. Our data suggests that Bcl-xL overexpression plays a role in castration-resistant progression of prostate cancer. Bcl-xL-targeted therapy in conjunction with androgen ablation might provide additive benefit for castration-resistant diseases.

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ORIGINAL ARTICLE

Phosphoinositide 3-OH kinase p85α and p110β are essential for androgen receptor transactivation and tumor progression in prostate cancers

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Phosphoinositide 3-OH kinases (PI3Ks) are a group of major intracellular signaling molecules. In our previous study, we found that inhibition of PI3K activity suppressed the androgen receptor (AR)-mediated gene expression in prostate cancer cells. The AR has been considered as a critical determinant for the development and progression of human prostate cancers. In this study, we sought to identify the PI3K isoforms involved in AR transactivation. Using a gene-specific small interference RNA (siRNA) approach, we determined that the regulatory isoform p85α and the catalytic isoform p110β, but not p110α, were required for androgen-stimulated AR transactivation and cell proliferation in prostate cancer cells. Consistently, overexpression of wild-type p110β but not p110α gene led to androgen-independent AR transactivation. Silencing p110β gene in prostate cancer cells abolished tumor growth in nude mice. Of the dual (lipid and protein) kinase activities, p110β's lipid kinase activity was required for AR transactivation. Further analysis by a chromatin immunoprecipitation assay showed that p110β is indispensable for androgen-induced AR–DNA interaction. Finally, gene expression analysis of clinical specimens showed that both p85α and p110β were highly expressed in malignant prostate tissues compared to the nonmalignant compartments, and their expression levels correlated significantly with disease progression. Taken together, our data demonstrated that p85α and p110β are essential for androgen-stimulated AR transactivation, and their aberrant expression or activation might play an important role in prostate cancer progression.
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Keywords: prostate cancer; PI3K; androgen receptor; siRNA; tumor progression; mouse xenograft

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Introduction

Phosphoinositide 3-OH kinases (PI3Ks) regulate multiple cellular signal pathways (reviewed in Bader *et al*., 2005; Engelman *et al*., 2006). In mamammlian cells, there are three classes (I, II and III) of PI3K isoforms, and the class I PI3Ks are further divided into two subtypes (IA p110 α/β/δ and IB p110γ), of which IA catalytic isoforms are associated with p85 regulatory subfamily, while IB p110γ is associated with p101/p84 regulatory subunit. The classes I and III but not class II PI3Ks are dual functional kinases, namely lipid kinase and protein kinase (Engelman *et al*., 2006).

Androgens are essential not only in the physiological development of male sexual phenotype but also in the genesis of prostate cancer. Upon its entry into target cells, the androgenic hormone transmits its regulatory signal to the nucleus through its cognate androgen receptor (AR), which is a ligand-activated transcription factor. After androgen stimulation, the AR itself and its co-factors are modified by post-transcriptional mechanisms (that is, phosphorylation, acetylation and so on), and growth factor-induced intracellular signaling pathways were reported to activate the AR independent of the androgens or to sensitize the AR to reduced levels of hormones, leading to hormone refractory (or so-called castration-resistant progression, reviewed in Scher and Sawyers, 2005).

Due to the inactivating mutation of PI3K signaling opponent *PTEN* gene (phosphatase and tensin homologue deleted on chromosome 10; Li *et al*., 1997; Steck *et al*. 1997), or through unknown mechanism after androgen withdrawal, elevated PI3K activity has been proposed as one of the major mechanisms for prostate cancer progression (Murillo *et al*., 2001; reviewed in Majumder and Sellers, 2005). Previous studies from our group (Liao *et al*., 2004) and others (Li *et al*., 2001; Sharma *et al*., 2002) have shown that PI3K activity is indispensable for androgen-induced AR transactivation in prostate cancer cells. However, it is not clear which PI3K isoforms participate in androgen-induced AR transactivation and castration-resistant progression in prostate cancers.

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To understand the mechanisms involved in prostate cancer progression, in this study, we went on to determine the PI3K isoforms that are required for androgen-induced AR transactivation and gene expression. We determined that PI3K class IA p85α and p110β isoforms are essential for androgen-stimulated AR transactivation and cell proliferation. We also found that these two isoforms are highly expressed in malignant prostate tissues compared to the nonmalignant compartments, and their expression levels correlate significantly with disease progression of prostate cancers.

Results

PI3K p85α and p110β are essential for androgen-induced AR transactivation and cell proliferation

Although PI3K has been long considered as an important factor for prostate cancer progression (Murillo *et al*., 2001; reviewed in Majumder and Sellers, 2005) and PI3K activity is required for AR-mediated gene expression (Liao *et al*., 2004), the PI3K isoforms responsible for this activity were not determined. Therefore, in this study, we used the small interference RNA (siRNA) approach to determine which PI3K isoforms are involved in AR transactivation. We focused on three ubiquitously expressed class IA isoforms: p85α, p110α and p110β. The efficiency and the specificity of the siRNAs against the corresponding PI3K isoforms were verified in prostate cancer LNCaP cells (Figure 1a). Meanwhile, no obvious effect of these PI3K siRNAs on AR expression was noticed (Figure 1b). Then, an androgen-responsive reporter construct (ARE-LUC) was used to evaluate the effect of PI3K siRNAs on androgen-induced AR transactivation. As shown in Figure 1c, transfection with the siRNAs for p85α and p110β but not p110α significantly suppressed R1881 (a synthetic androgen)-induced ARE-LUC activity. Since there was a reduction in ARE-LUC activity in p110α siRNA-transfected cells, we used PI3K isoform-specific inhibitors to verify if p110β is unique for AR-mediated gene expression. The isoform specificity of the inhibitors, PI3K Inhibitor 4 (PI3K-I4) for p110α and TGX-221 for p110β, was described previously (Jackson *et al*., 2005; Hayakawa *et al*., 2006). The mRNA expression level of the well-known AR target *psa* gene was chosen to evaluate the effect of these inhibitors on androgen-stimulated AR transactivation. Consistent with the data from the siRNA experiments (Figure 1c) and our previous report (Liao *et al*., 2004), pretreatment with LY294002 (a nonspecific PI3K inhibitor) or TGX-221 but not PI3K-I4 significantly suppressed R1881-stimulated prostate-specific antigen (PSA) expression (Figure 1d). These data indicated that type IA p85α and p110β but not p110α are required for androgen-induced AR transactivation.

To validate the biological significance of these PI3K isoforms' involvement in androgen action, we utilized two different assays (MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and bromodeoxyuridine (BrdU) incorporation) to determine the effect of PI3K siRNAs on androgen-stimulated LNCaP cell proliferation. Consistent with the results from ARE-LUC reporter gene assay (Figure 1c), transfection with the siRNAs for p85α and p110β but not for p110α significantly suppressed R1881-stimulated cell proliferation in LNCaP cells (Figures 1e and f). These data clearly demonstrated that p85α and p110β are required for androgen-stimulated cell proliferation of prostate cancer *in vitro*.

PI3K p110β is required for tumor growth in vivo

Then, we extended our *in vitro* cell-based studies into an *in vivo* system. LNCaP-derived mouse xenograft model was used to test if PI3K p110β is required for tumor growth. LNCaP subline LNCaP/p110βshRNA cells were established by stable transfection with a p110β siRNA-expressing construct (Czauderna *et al*., 2003) and were used to generate the xenografts in nude mice. The LNCaP/Neo subline cells transfected with a plasmid vector harboring the neomycin-resistant gene were used as the control. The efficiency of the p110β silencing construct on p110β expression was confirmed by western blot assay (Figure 2a), and no. 3 subline cells were then used for the xenograft experiments. As expected, when LNCaP/Neo cells were subcutaneously allocated into nude mice, an 80% tumor development rate (8 out of 10 allocations) was achieved after 6 weeks (Figure 2b; image data not shown), which was consistent with our recent report (Li *et al*., 2007b). In contrast, no tumor development was observed in LNCaP/p110βshRNA cells-allocated animals even after 8 weeks. These data indicated that p110β is essential for tumor growth of LNCaP xenograft *in vivo*.

PI3K p110β overexpression induces androgen-independent AR transactivation

Previous studies showed an association between increased PI3K activities and androgen-independent progression of prostate cancers (Murillo *et al*., 2001; reviewed in Majumder and Sellers, 2005). Thus, we tested if overexpression of p110α or p110β enhances androgen-stimulated AR transactivation or even induces androgen-independent AR transactivation. First, LNCaP cells were transfected with p110α- or p110β-expressing constructs together with the ARE-LUC reporter. Cells were then treated with increasing amount of R1881 or the solvent as control. As shown in Figure 3a, p110β overexpression not only significantly enhanced R1881-induced ARE-LUC reporter activity, but also activated ARE-LUC reporter in the absence of R1881. However, p110α overexpression did not affect R1881-induced ARE-LUC activity. A dose–response effect was clearly seen when increasing amounts of p110β constructs were used in the transfection experiments (Figure 3b). These data indicated again that p110β but not p110α enhances AR activity in the presence of castration-level androgens or even in the absence of androgens. It is plausible that elevated

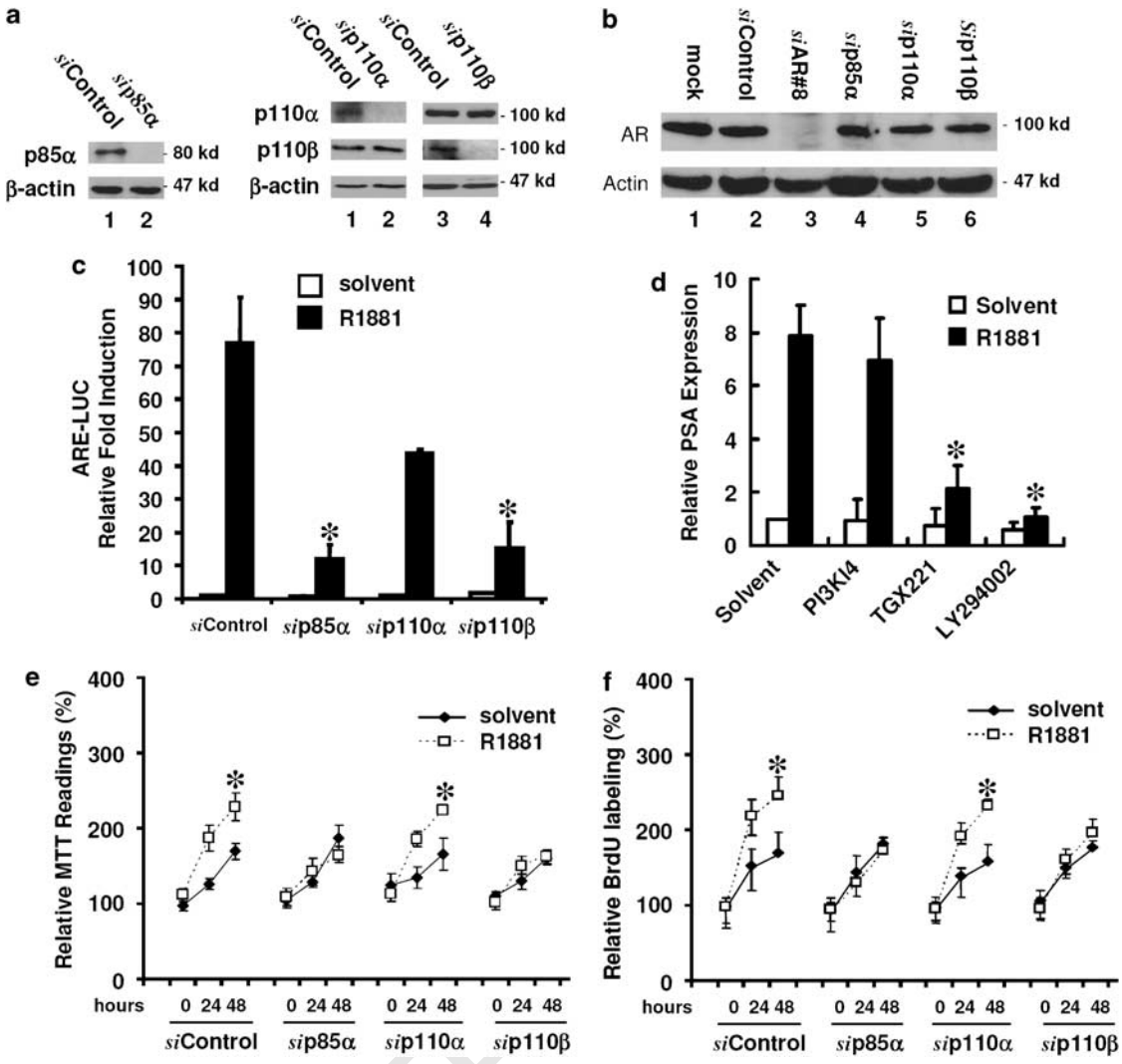

Figure 1 Phosphoinositide 3-OH kinases (PI3K) p85α and p110β isoforms are essential for androgen-induced androgen receptor (AR) transactivation and cell proliferation. (**a**) The efficiency of small interference RNA (siRNA)-induced gene silencing on PI3K isoforms was evaluated and (**b**) the effect of PI3K siRNAs on AR expression in LNCaP cells. Equal amount of proteins from cells transfected with the siRNAs for individual PI3K isoforms, no. 8 AR siRNA or a negative control siRNA were subjected for western blot. Mock transfection was conducted by adding transfection reagent only. Anti-actin blot served as protein loading control. Molecular weight markers were positioned on the right side of each panel. (**c**) The effect of PI3K siRNA on AR transactivation. After transfection with the siRNAs as indicated for 2 days, LNCaP cells were co-transfected with a luciferase reporter construct controlled by synthetic androgen response elements (ARE-LUC) and CMV-secreted alkaline phosphatase (SEAP) reporter constructs. Cells were treated with the solvent or R1881 (1.0 nM) in a media supplied with 2% charcoal-stripped FBS (cFBS), and the media were collected for SEAP assay and cell lysates were subjected for luciferase assay. Average fold induction against control transfection was calculated after normalized against the corresponding SEAP activity and protein concentration. (**d**) The effect of PI3K isoform-specific inhibitors on androgen-stimulated prostate-specific antigen (PSA) expression. LNCaP cells were serum starved for 24 h and then pretreated with the inhibitors (PI3K-I4 at 100 nM, TGX-221 at 500 nM and LY294002 at 25 μM) as indicated for 45 min. After R1881 treatment for 8 h, cells were harvested for total RNA extraction. PSA expression at mRNA level was determined by real-time reverse transcription (RT)–PCR. The expression levels of epithelium-specific gene *KRT18* was used to normalize *psa* expression values. Data were from three independent experiments. Error bars represent standard error (s.e.) and the asterisk (*) indicates a significant difference ($P<0.05$, Student's *t*-test). (**e**, **f**) After transfection with the siRNAs as indicated for 2 days, LNCaP cells were treated with the solvent or R1881 for up to 48 h in 5% cFBS-containing media. Cell proliferation rate was determined everyday with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (**e**) and bromodeoxyuridine (BrdU) incorporation (**f**) assays as described in the text. Data represent the average percentage of 24 and 48 h over 0 h from two independent experiments. Hour 0 indicates the starting date of treatment and the value was set as 100%. The asterisk (*) indicates a significant difference compared to the solvent control ($P<0.05$, Student's *t*-test).

expression or overactivation of p110β isoform in prostate cancer cells may activate the AR after androgen withdrawal.

It has been shown that p110β possesses both lipid and protein kinase activities (reviewed in Bader *et al*., 2005; Engelman *et al*., 2006). To determine which activity of p110β kinase is involved in AR transactivation, we tested the effect of different p110β mutants, a total kinase-dead mutant (K805R) and a protein kinase only mutant (PKO, lipid kinase deficient) on androgen-

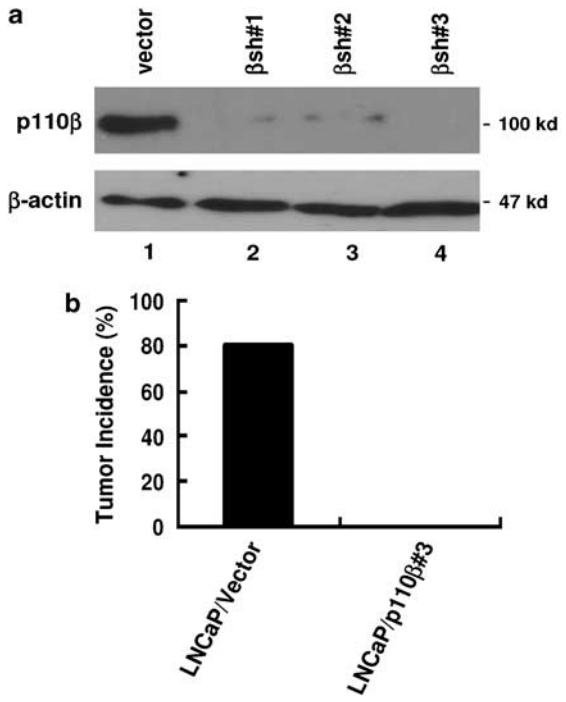

Figure 2 Knocking down phosphoinositide 3-OH kinases (PI3K) p110β expression abolishes tumor growth of LNCaP cell-derived xenograft in nude mice. (**a**) LNCaP cells were transfected with pU6+2/p110βshRNA vector, and stable clones (LNCaP/p110βshRNA) were selected in G418-containing media. An empty vector was used to establish the control subline (LNCaP/vector). The efficacy of p110β silencing in representative sublines was evaluated by western blot assay. (**b**) Single cell suspension of the LNCaP/vector and LNCaP/p110βshRNA no. 3 sublines at a dose of 2×10^6 cells per 0.1 ml media were inoculated subcutaneously into the rear flanks of 6- to 7-week-old male nude mice. Xenograft tumor growth was monitored every day. The percentage of tumor incidence was shown from each group.

induced ARE-LUC activity. The efficacy of these p110β mutants was verified previously (Yart *et al*., 2002). As shown in Figure 3b, while the wild-type p110β construct significantly enhanced AR transactivation not only in the presence of androgen but also in the absence of androgen, overexpression of the K805R and PKO mutants both dramatically reduced androgen-induced ARE-LUC activity. These results suggested that the lipid kinase activity is responsible for p110β-mediated regulation of AR transactivation.

PI3K p110β is required for AR–DNA interaction

We previous showed that PI3K inhibitor suppressed AR-mediated gene expression but did not block AR nuclear translocation (Liao *et al*., 2004), indicating that PI3K signaling might be involved in the assembly of AR-mediated transcription complex including AR–DNA interaction. Therefore, we performed a chromatin immunoprecipitation (ChIP) assay to determine if p110β is required for AR interaction with chromatin DNA. The well-known AR-regulated gene *psa* promoter was used as the target sequence, and the primers were adapted from a recent publication (Shang *et al*., 2002). We first performed a pilot experiment to clarify the specificity of the procedure. As shown in Figure 4a, the antibodies for RNA polymerase II (Poly II) but not the normal serum pulled down the housekeeping glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene promoter, as detected by the PCR reaction. Then, we conducted the anti-AR ChIP assay for *psa* promoter. As shown in Figure 4b, R1881 treatment induced AR interaction with the *psa* promoter, which is abolished by pretreatment of PI3K inhibitor Wortmannin. However, Wortmannin treatment did not affect Poly II binding to the *GAPDH* gene promoter, confirming the specificity of PI3K activity for AR transactivation. These data indicated that PI3K activity is required for AR–DNA interaction.

Next, we examined the effect of p110β siRNAs on AR–DNA interaction. In these experiments, we included a primer pair (rtPSA) that was used in our previous report for reverse transcription (RT)–PCR experiments (Liao *et al*., 2005) as a negative control. As shown in Figure 4c; no PCR products were obtained when the rtPSA primers were used, confirming the assay's specificity for AR–DNA (*psa* promoter) binding. Transfection of p110β siRNAs but not the control siRNA abolished androgen-induced AR interaction with *psa* promoter. Taken together, these data clearly demonstrated that p110β-derived signaling is essential for androgen-induced AR–DNA binding.

PI3K p110β expression increases along with prostate cancer progression

To establish the clinical relevance of p110β involvement in AR activation and prostate cancer progression, we examined the expression levels of PI3K isoforms in human prostate cancers. First, we re-analysed a published cDNA microarray data set (Holzbeierlein *et al*., 2004), of which the cDNA microarray experiments were conducted on an Affymetrix HG-U95 chip. There were five groups of prostate specimens from normal prostates, primary cancers, tumors after hormone therapy, metastatic tumors and hormone-refractory tumors. As shown in Figure 5a, the expression levels of p110β (*PIK3Cb*) but not p110α and p110γ was dramatically increased in prostate cancers, including the aggressive metastatic and hormone-refractory tumors compared to that in normal tissues. Most significantly, these data also showed a positive correlation between p110β expression and disease progression.

To verify the cDNA microarray data, we quantitatively examined the expression levels of PI3K genes using real-time RT–PCR approach. A total of 30 primary tumor samples plus the individually matched nonmalignant compartments in each case were obtained from our tissue repository. These prostate specimens were obtained from patients with primary cancers at surgery (radical prostectomy). The pathology of each tissue sample was examined by haematoxylin and eosin (H&E) staining on a representative frozen section. Since the heterogeneous feature of prostate cancer tissue, we used the expression levels of an epithelial cell-specific gene *KRT18*, as described (Latil *et al*. 2001), to

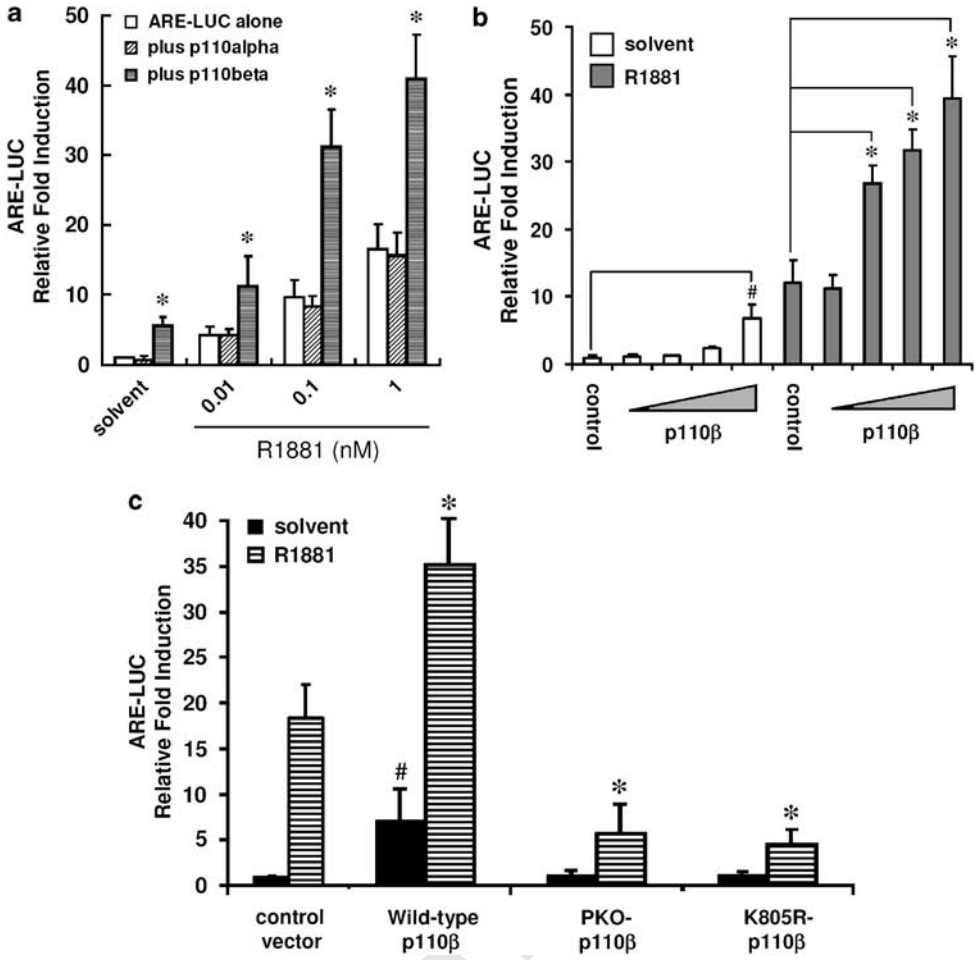

Figure 3 Phosphoinositide 3-OH kinases (PI3K) p110β overexpression results in androgen-independent androgen receptor (AR) transactivation. LNCaP cells were transfected with wild-type p110α- or p110β-expressing constructs at 1.5 μg per well (**a**) or with increasing amount (0.25, 0.5, 1.0, 1.5 μg) of p110β constructs (**b**) together with the luciferase reporter construct controlled by synthetic androgen response elements (ARE-LUC) and CMV-secreted alkaline phosphatase (SEAP) reporters. Control cells received pcDNA3.1 empty vector (1.5 μg) for equalizing the DNA amount used. After serum starvation, cells were treated with the solvent, increasing doses of R1881 as indicated (**a**) or R1881 at 1.0 nM (**b**) for 24 h in 2% charcoal-stripped fetal bovine serum (cFBS)-containing media. Luciferase and SEAP activities were assessed and data were presented as described earlier. (**c**) LNCaP cells were transfected with wild-type and mutant p110β constructs (1.5 μg) together with the ARE-LUC and CMV-SEAP reporters. After serum starvation for 24 h, cells were treated and reporter assays were conducted as described above. The error bars represent standard error of the mean (s.e.m.) and the asterisk (*) indicates a significant difference ($P<0.05$, Student's *t*-test) between R1881 treatment and the solvent control. The # sign indicates a significant difference ($P<0.05$) between wild-type p110β and the vector control in the absence of R1881.

normalize the expression levels of PI3K isoform genes. In 33.3% (10 out of 30) of cases, their p85α expression levels were more than twofold higher in tumor samples compared to that in the nonmalignant compartments (Figure 5b). For 46.7% (14 out of 30) of cases, p110β expression showed a twofold or more increase in malignant tissues compared to the nonmalignant compartments (Figure 5c). The differences in the average levels of p85α and p110β were significant between the malignant tissues and their nonmalignant compartments (Figure 5d). However, there was not significant difference for the expression levels of p110α and p110γ between malignant tissues and nonmalignant compartments. Meanwhile, p110γ exhibited the lowest expression levels among the PI3K isoforms examined, which was consistent to previous reports (reviewed in Bader *et al*., 2005; Engelman *et al*., 2006).

To test if p85α expression correlates with disease progression of prostate cancers, we examined p85α protein expression using immunohistochemical (IHC) staining approach. A total of 30 paraffin-embedded prostate cancer specimens from primary tumors with various grades (Gleason score) and metastatic tumors were used in this experiment. The immunosignal intensities were scored as weak, moderate and strong, and the representative images for this scoring system were presented in Figure 6A. On the basis of the sum of Gleason score, the primary tumors were divided into three groups; low (G5–6), medium (G7) and high (G8–9). As shown in Figure 6B, almost all cases (8 out of 9) of low-grade tumors showed a weak signal of p85α expression, while 60% (6 out of 10) of high-grade tumors showed strong signals for p85α expression. In metastatic tumors, 77.8% (7 out of 9) of the cases

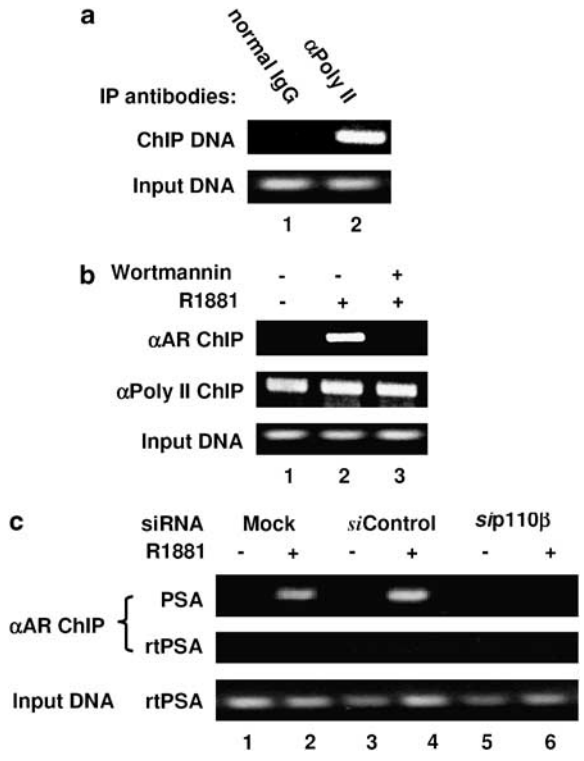

Figure 4 Phosphoinositide 3-OH kinases (PI3K) signaling modulates androgen receptor (AR)–DNA binding. (**a**) Exponential grown LNCaP cells were fixed and harvested for a pilot chromatin immunoprecipitation (ChIP) assay with non-immunonized rabbit immunoglobulin G (IgG) and anti-RNA polymerase II antibodies as described in the text. The PCR reaction was performed with a primer pair for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene promoter. (**b**) Serum starved LNCaP cells were pretreated with the solvent or PI3K inhibitor Wortmannin (1.0 μM) for 45 min followed by R1881 (1.0 nM) as indicated for 12 h in 5% charcoal-stripped FBS (cFBS). Cells were fixed and harvested for anti-AR and anti-Poly II ChIP assay. PCR reaction was conducted with the primers for prostate-specific antigen (*psa*) gene promoter (anti-AR ChIP) or GAPDH gene promoter (anti-Poly II ChIP). (**c**) LNCaP cells were transfected with the small interference RNAs (siRNAs) as indicated for 3 days and mock transfection was included as a negative control. After treatment with the solvent or R1881 in 5% cFBS for 12 h, cells were fixed and harvested for anti-AR ChIP assay with the primer pairs for *PSA* gene promoter (PSA primer) and PSA gene coding region (rt-PSA primer) as described in the text. For all the panels, 1% of the input chromatin was used as template in PCR reaction as the positive control of the primers used. Data represent two independent experiments.

showed a strong signal for p85α expression. The difference in p85α expression was significant ($P<0.05$, χ^2-test) between the higher-grade tumors (G8–9 and Met) and the lower grade tumors (G5–6 and G7). These data suggested that elevated expression of p85α is associated with prostate cancer progression.

Discussion

Although PI3K activity has been considered as an important factor in AR-mediated gene expression and prostate cancer progression, it is not clear which isoforms are involved in this process. In this study, we provided convincing evidence for the first time that PI3K isoforms p85α and p110β are essential for androgen-induced AR transactivation, cell proliferation and tumor growth. Knocking down PI3K p110α did not result in any significant effect on AR transactivation and cell proliferation, which is supported by a previous report (Czauderna *et al*., 2003). Most importantly, we demonstrated the involvement of p110β in androgen-induced AR interaction with chromatin DNA, a key step for AR regulation of gene expression. We also determined that p110β overexpression led to androgen-independent AR transactivation, and that the expression levels of p85α and p110β genes are significantly higher in malignant prostate tissues compared to their nonmalignant compartments. Our studies suggest that aberrant expression of p85α/p110β isoforms might play an important role in androgen-independent (or so-called castration-resistant) progression of prostate cancers.

There are two major steps for hormone-bound AR to exert its genomic effect as a transcription factor; nuclear translocation and chromatin DNA binding. Currently, it is not clear how precisely androgen induces AR nuclear translocation. Once the AR is in the nucleus, it will assemble a mega-protein transcription complex, which is a dynamic process including transient recruitment and then dissociation of co-factors, chromatin remodeling molecules, proteasome subunits and general transcription machinery (reviewed in Heinlein and Chang, 2004). We recently showed that PI3K inhibitors could not block AR nuclear translocation but suppressed AR-mediated gene expression (Liao *et al*., 2004), indicating that a PI3K-dependent mechanism is involved in AR–DNA binding, or the assembly of AR-mediated transcription complex. Indeed, in this study, we found that PI3K inhibitor abolished androgen-induced AR–DNA binding. Knocking down p110β expression also resulted in loss of AR–DNA binding. A similar effect was also reported by another group using Her2 inhibitor (Mellinghoff *et al*., 2004). Our studies indicate that p110β signaling, most likely coupling with Her2 kinase, is required for AR–DNA interaction or the dynamic assembly of AR transcription complex.

Recent emerging evidences indicate that PI3K isoforms have oncogenic potential through gain-of-function mutations or gene amplification (Zhao *et al*., 2005; reviewed in Bader *et al*., 2005). Because of their important roles in cell signaling, elevated activities of PI3K pathway due to PTEN inactive mutation has long been considered as a key player in cancer pathogenesis including prostate cancers (Li *et al*., 1997; Steck *et al*., 1997; Murillo *et al*., 2001; reviewed in Engelman *et al*., 2006). However, no information is currently available about the genetic phenotype and expression pattern of PI3K isoforms in human prostate cancers. Only one report showed that siRNA-mediated p110β silencing suppressed cancer cell growth *in vitro* and tumor metastasis *in vivo* (Czauderna *et al*., 2003). In this study, we showed that both p85α and p110β are highly expressed in prostate cancers at the mRNA and protein levels, respectively. Their expression levels are associated

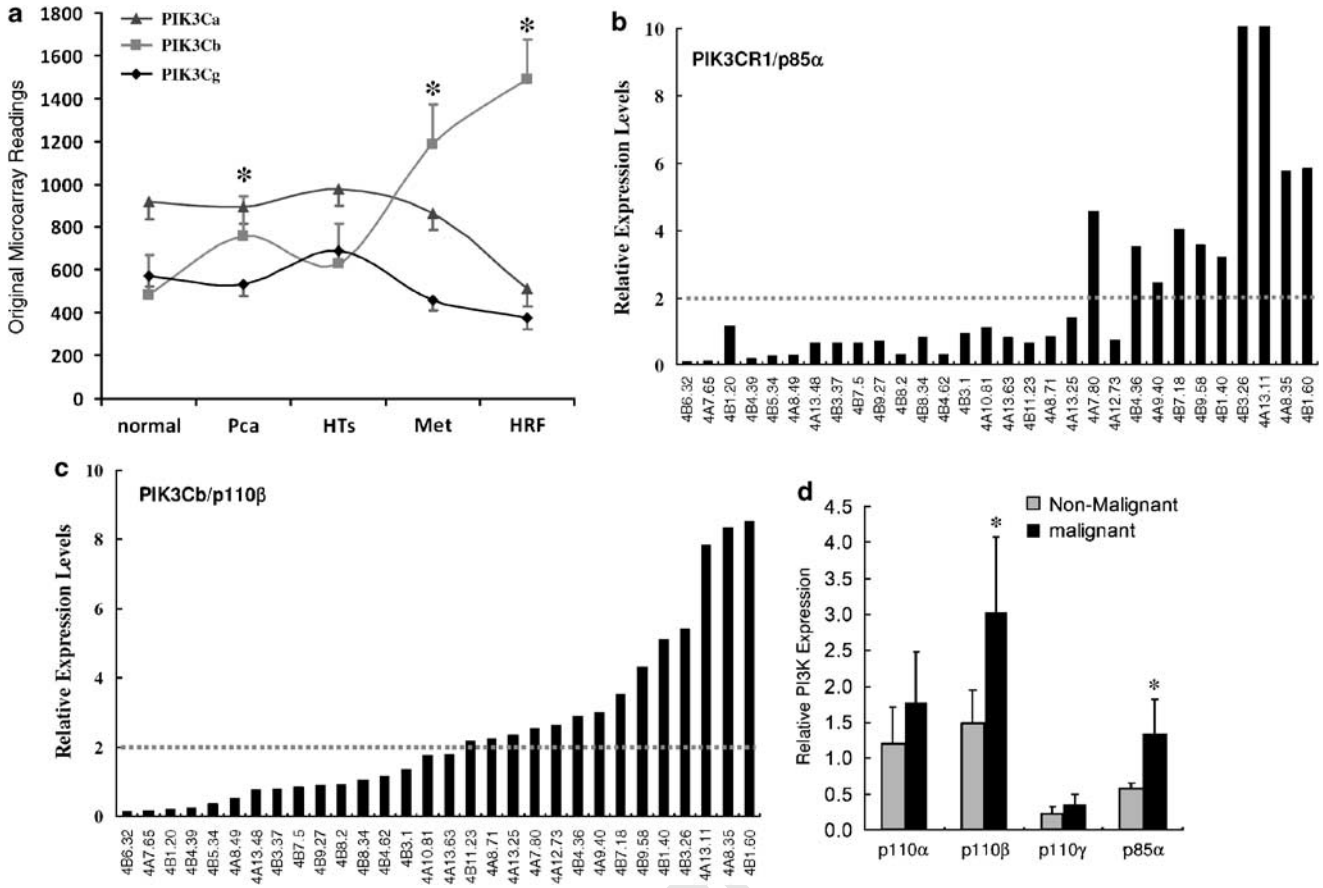

Figure 5 Phosphoinositide 3-OH kinases (PI3K) p85α and p110β isoforms are overexpressed in human prostate cancers. (**a**) Raw data (mean) from a published cDNA microarray data set were graphed based on different patient groups, including normal prostate specimens (normal, $n=5$), primary tumors (Pca, $n=23$), tumors after hormone therapy (HTs, $n=17$), metastatic tumors (Met, $n=9$) and castration-resistant tumors (HRF, $n=3$). Error bar represents the standard error of mean (s.e.m.). The asterisk (*) indicates a significant difference compared to the 'normal' group ($P<0.05$, Student's *t*-test). *PIK3Ca*, p110α; *PIK3Cb*, p110β; *PIK3Cg*, p110γ. (**b**–**d**) Quantitative analysis of PI3K isoforms in prostate cancers. Total RNAs were extracted using a TriZol-based protocol from frozen tumor specimens and the individually matched nonmalignant compartments. Gene expression at mRNA level was assessed by real-time reverse transcription (RT)–PCR. The expression levels of PI3K genes were normalized against the epithelium-specific gene *KRT18* before the relative values were calculated. The relative ratio of gene expression level in malignant compared to nonmalignant tissues in each case was presented in (**b**) (p85α) and (**c**) (p110β). The average levels of PI3K genes were summarized in (**d**). Error bar represents the standard error of mean (s.e.m.), and the asterisk (*) indicate a significant difference ($P<0.05$, Student's *t*-test) compared to nonmalignant tissues.

with disease progression, poor differentiation and metastasis. Although the patient population was limited, our findings are of clinical significant because it opens a novel area for further investigation in prostate cancers.

Usually, type IA PI3K is activated by membrane-associated tyrosine kinase-based mechanisms through p85 regulatory isoforms. On the other hand, Gα subunits-mediated p110β activation was also reported (Murga *et al*., 1998; reviewed in Bader *et al*., 2005). A recent report showed that androgen induces PI3K activation *via* AR interaction with p85α and Src kinase in prostate cancer cells (Sun *et al*., 2003). However, we (Liao *et al*., 2004) and other (Mellinghoff *et al*., 2004) found that Src kinase inhibitor did not suppress androgen-induced gene expression, indicating that Src kinase is not involved in androgen-induced PI3K activation or AR genomic effect. In addition, recent studies showed that after androgen stimulation the AR was transiently translocated to the lipid rafts on the plasma membrane (Lu *et al*., 2001; Cinar *et al*., 2007), where multiple signaling molecules such as G proteins are located (reviewed in Chini and Parenti, 2004). In fact, G protein α-subunit has been shown to activate the AR in the absence or presence of androgens (Kasbohm *et al*., 2005). Collectively, it is plausible that p85α and/or G proteins are involved in androgen-stimulated p110β activation and that in turn induces AR transactivation.

In conclusion, we demonstrated that PI3K p85α and p110β are the essential signaling components in androgen-induced AR transactivation and are required for cell proliferation and tumor growth of prostate cancer. It is plausible that these PI3K isoforms or other signaling molecules, such as Her2 kinases (Mellinghoff *et al*., 2004), are working in concert to regulate AR–DNA interaction or assembly of AR-based transcriptional complex on the promoter region of target genes. Further

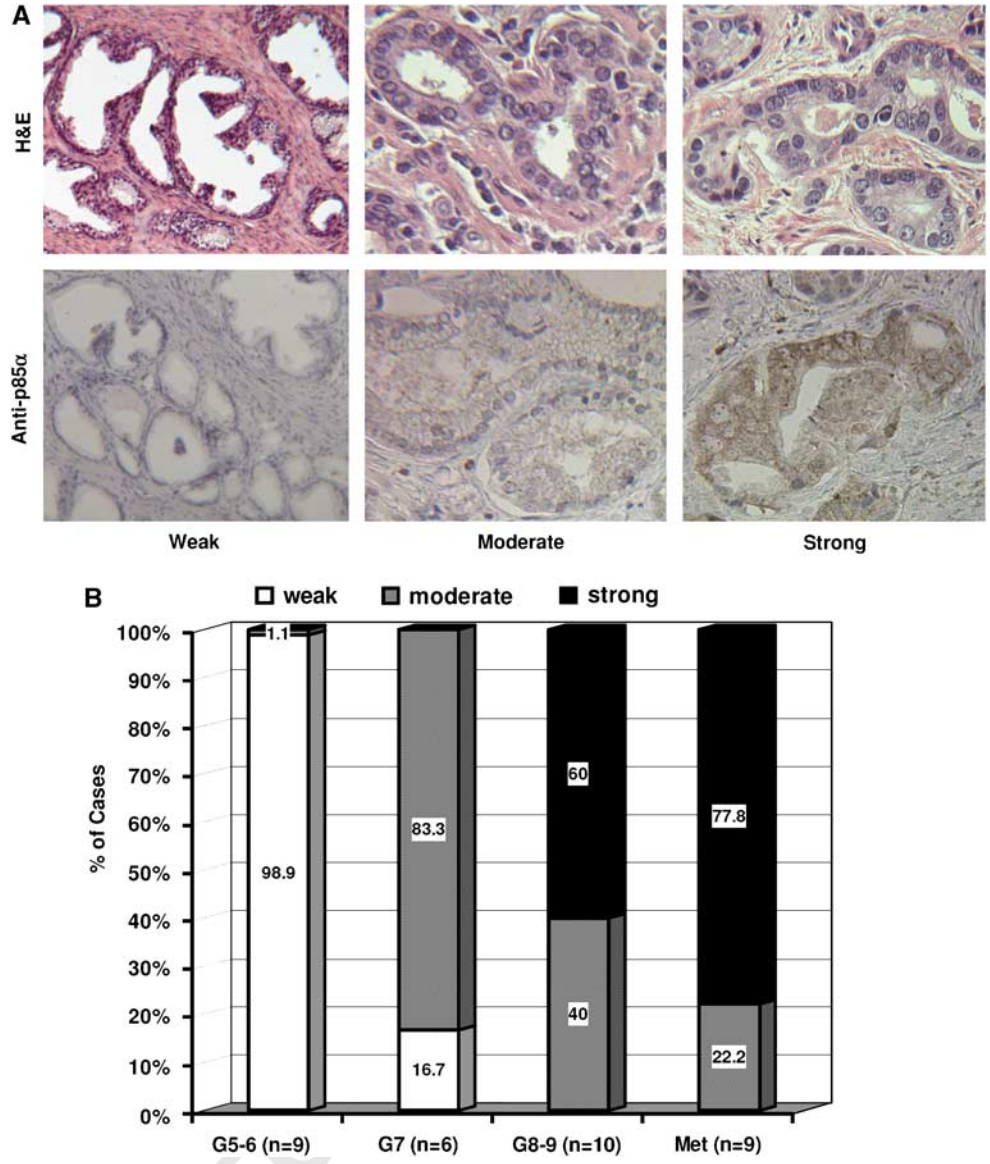

Figure 6 Increased p85α expression correlates with prostate cancer progression. (**A**) Representative micrographics for anti-p85α immunostaining and haematoxylin and eosin (H&E) staining in benign tissues (a, d) and malignant tissues (c–f). Positive staining was defined as a defused pattern of brown color. Magnification, (a, b) $\times 100$; (c–f) $\times 200$. (**B**) Case distribution of p85α immunostaining intensities in primary tumors and metastatic tumors (Met). Semiquantitative scoring of immunosignals was conducted as described in our recent report (Sun *et al*., 2007b). Labeled values on each column represent the percentage of positive cases in the corresponding category as marked. The *n* number indicates the case number in each group.

investigation is desirable for dissecting this complicated process in detail.

Materials and methods

Cell culture and reagents

The human prostate cancer LNCaP cell line and human embryonic kidney cell line HEK293 were described previously (Liao *et al*., 2004). The synthetic androgen R1881 was obtained from ICN (Aurora, OH, USA). TGX-221 was purchased from Cayman Chemical (Ann Arbor, MI, USA). LY294002 and p110α inhibitor 4 (PI3K-I4) were purchased from Calbiochem (San Diego, CA, USA). Antibodies for PI3K isoforms and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-AR antibodies (Clone PG21) were obtained from Upstate (Charlottesville, VA, USA). Charcoal-stripped fetal bovine serum (cFBS, steroid-depleted) was obtained from Atlanta Biologicals (Norcross, GA, USA). The mammalian expression constructs for wild-type p110β and its mutants were described previously (Yart *et al*., 2002), and p110α construct was obtained from OriGene (Rockville, MD, USA).

Western blot analysis

For western blot analysis, cells were washed in phosphate-buffered saline and lysed in a radioimmunoprecipitation assay buffer supplied with a cocktail of protease inhibitors (Cyto-Signal, Irvine, CA, USA). Equal amount of proteins was separated on an 8–15% SDS–polyacrylamide gel and blotted onto a polyvinyl difluoride membrane (Bio-Rad Laboratories,

Hercules, CA, USA). Membranes were blocked in a Tris-buffered saline solution with 5% nonfat dry milk containing 0.1% Tween-20 and incubated with antibodies overnight at 4 °C. Immunoreactive signals were detected by incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) followed by chemiluminescent detection using SuperSignal substrate (Pierce Chemical Co., Rockford, IL, USA).

siRNA transfection

The control siRNAs with scrambled sequences were purchased from Ambion (Austin, TX, USA) and the predefined siRNAs for PI3K isoforms were purchased from Santa Cruz Biotechnology. The no. 8 siRNA for human *AR* gene was described previously (Liao *et al*., 2005). Transfection was carried out with OligoFectamine reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. For the experiments involving a reporter gene assay, 3 days after transfection with the siRNAs, cells were transfected with the reporter constructs as described below. To determine the efficiency of the siRNAs, preliminary experiments were conducted in HEK293 and LNCaP cells by transfection of the siRNAs at various concentrations (1–100 nM), and a dramatic reduction was observed when the siRNAs were used at a final concentration of 100 nM in culture media. The scrambled siRNA duplex was used as a negative control.

Luciferase and SEAP reporter assay

A luciferase reporter construct controlled by synthetic androgen response elements (ARE-LUC) and the control reporter construct CMV–SEAP, expressing secreted alkaline phosphatase (SEAP) under the control of the cytomegalovirus (CMV) promoter, were described previously (Lee *et al*., 2003; Shanmugam *et al*., 2007). For PI3K overexpression experiments, cells were plated in six-well tissue culture plates and co-transfected on the following day with 1.0 μg ARE-LUC, 0.5 μg CMV-SEAP and various amount of PI3K constructs as indicated in the figure using the LipoFectamine reagent (Invitrogen). A pcDNA3.0 empty vector was used to balance the DNA amounts used in each transfection. After 24 h, cells were serum starved for another 24 h and then treated with the solvent (ethanol) or R1881 (1.0 nM) in 2% cFBS. After 24 h, culture supernatants were collected and assayed for SEAP activity as described previously (Liao *et al*., 2004). Cells were lysed with a luciferase assay lysis buffer (Promega Corp., Madison, WI, USA). Protein concentration of cellular lysate was measured by the BAC protein assay (Bio-Rad Laboratories). Equal amount of proteins from each cell lysate was assayed in triplicate for luciferase enzyme activity by using the luciferase assay system (Promega Corp). The luciferase activity of each sample was normalized against the corresponding SEAP activity and the protein concentration before the fold induction value relative to control cells was calculated (Liao *et al*., 2004).

Frozen tissue and real-time RT–PCR

A total of 30 primary prostate cancer specimens and the individually matched, microscopically confirmed nonmalignant compartments were obtained from patients who received surgery at the KU Hospital. A research protocol with the exemption of human subject was approved by the Institutional Research Board. After H&E staining, each tumor specimen was examined by an experienced surgical pathologist for the diagnosis (grade and stage) following the criteria of the American joint committee on cancer, 1988 edition. Tumor samples that contain at least 70% or more malignant compartments were selected for further experiments. Specimens (80–100 mg per sample) were snap frozen and stored at −80 °C before use. There were 24 cases with Gleason score of 7, 1 case with Gleason score of 6 and others were Gleason score 8–9. All patients were naive for hormonal therapy and the average of patient age was 62.5 years (57–78 years).

Total RNAs from frozen tissues and LNCaP cells after treatment with PI3K inhibitors were extracted using TriZol reagent (Invitrogen). The first-strand cDNA was synthesized using Omniscript-RT Kit (QiaGen, Valencia, CA, USA). The primers for the genes of *PIK3Ca* (p110α; 5′-ATCTTTTCT-CAATGATGCTTGGCT-3′ and 5′-CTAGGGTGTTTC-GAATGTATG-3′), *PIK3Cb* (p110β; 5′-CCCTTCTGAA CTGGCTTAAAGA-3′ and 5′-GGACAGTGTAAATTCCT-CAATGG-3′), *PIK3R1* (p85α; 5′-AATGAACGACAGCCT GCAC-3′ and 5′-CCGTTGTTGGCTACAGTAGTAGG-3′), as well as for *psa* (5′-ACCAGAGGAGTTCTTGACCC-CAAA-3′ and 5′-CCCCAGAATCACCCGAGCAG-3′) were synthesized by IDT Inc (Coralville, IA, USA). The real-time PCR was performed using SYBR Green Supermix kit with the Bio-Rad iQ5 system. Denaturation at 95 °C for 15 s, and annealing/extension (temperature varied due to different primer sequence composition) for 1 min was then performed for 30 cycles. The amplification value of the target genes was normalized against the epithelial cell-specific gene *KRT18* as described (Latil *et al*., 2001).

Cell proliferation assay

Cells were seeded in 96-well microtiter plate overnight. Thereafter, cells were transfected with the siRNAs and then incubated for additional 24 and 48 h in the presence or absence of R1881. Cell proliferation rates in each cell line were analysed using MTT assay (Cell Proliferation kit I, Roche Molecular Biochemicals, Indianapolis, IN, USA). Formazan crystals were solubilized overnight, and the product was quantified spectrophotometrically at 570 nm.

For BrdU incorporation assay, cells were incubated with BrdU (BrdU labeling and detection kit III; Roche Molecular Biochemicals) for the last 18 h of the experiments. Cells were subsequently fixed with 0.5 M ethanol/HCl and were then incubated with nucleases to partially digest DNA. Monoclonal anti-BrdU antibodies conjugated to peroxidase were subsequently added and detected with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) as the substrate. Quantitation was performed colorimetrically at 405–490 nm acording to the manufacutrer's protocol.

ChIP assay

ChIP assay was conducted basically as described in our recent publications (Li *et al*., 2007a; Sun *et al*., 2007a). Cells were maintained in 10-cm dishes in medium without serum for at least 16 h. Following PI3K siRNA transfection for 3 days or addition of the PI3K inhibitor Wortmannin, cells were treated with or without 1.0 nM R1881 for 12 h in the presence of 5% cFBS. The ChIP assay kit and the polyclonal antibody against AR were obtained from Upstate. Normal rabbit serum was used as a negative control. An anti-RNA polymerase II antibody (Santa Cruz Biotechnology) was used as a positive control. GAPDH primers were supplied by the ChIP assay kit. The primers for *psa* gene in the PCR reactions were listed as follows: 5′-tctgcctttgtcccctagat-3′ and 5′-aaccttcattccccaggact-3′ (adapted from Shang *et al*., 2002), which amplify a 210-bp fragment corresponding to human *PSA* gene promoter sequence between −250 and −39 from the transcription start site. The PCR products were run on 1% agarose gel and stained with ethidium bromide for visualization.

LNCaP stable cell lines and mouse xenograft model
LNCaP cells were transfected with the pU6 + 2 vector bearing a hairpin-structured siRNA cassette for p110β silencing (pU6 + 2-p110β.shRNA), as described previously (Czauderna *et al*., 2003). Stable clones were selected in G418-containing media, and the control sublines were established as described (Liao *et al*., 2004; Shanmugam *et al*., 2007). The efficacy of p110β silencing was validated by western blot (Figure 2a). All animal studies were conducted under an approved institutional animal care and use committee protocol. Single cell suspension of 2×10^6 cells in 0.1 ml of RPMI 1640 medium plus MatriGel supplied with 10% FBS were inoculated subcutaneously into the rear flanks (two sites per mouse) of 6- to 7-week-old male nude mice (Charles River, Wilmington, MA, USA). Tumor development was monitored every other day.

Tissue samples and IHC staining
A total of 34 paraffin-embedded specimens from 25 primary cancers with different tumor grades (Gleason Score) and 9 metastasis tumors were selected for the IHC study, following a protocol approved by our Institutional Research Board. The Clinical data was collected at the time of selection. The histopathological data included the diagnosis at surgery (stage and grade, surgical margin, lymph note status), tumor volume, cell proliferation marker Ki-67, DNA content (ploidy) and p53 status. Patients were followed up for survival and disease recurrence. Mean patient age was 58.6 years and the average Gleason score was 7.4.

The IHC staining was conducted as described in our recent publication (Sun *et al*., 2007b). Briefly, the sections were deparaffinized and rehydrated, followed by antigen retrieval and endogenous peroxidase blocking. The primary antibodies for p85α (clone B-9, catalog no. SC1637) were purchased from Santa Cruz Biotechnology. Multiple dilutions of the primary antibodies were utilized for individual specimens to achieve optimal immunosignals. A negative control was set up for each case by omitting the primary antibody. The specificity of the primary antibodies was verified by western blot. Immunosignals were visualized with a DAKO LSAB kit (Dako, Carpenteria, CA, USA). The intensity of the immunosignals was scored as weak, moderate and strong.

Statistical analysis
All experiments were repeated two or three times except animal studies. Western blot and IHC staining results are presented from a representative experiment. The mean and standard error of the mean from multiple experiments for reporter gene assays and cell proliferation assays are shown. The significance of the differences between treatment and control was analysed using the SPSS computer software (SPSS Inc., Chicago, IL, USA).

Abbreviations

AR, androgen receptor; ARE, androgen response element; BrdU, Bromodeoxyuridine; ChIP, chromatin IP; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAGE, polyacrylamide; PI3K, phosphoinositide 3-OH kinase; PKO, protein kinase only; PSA, prostate-specific antigen; PTEN, phosphatase and tensin homologue deleted on chromosome 10; RT, reverse -transcription; SEAP, secreted alkaline phosphatase; s.e.m., standard error of mean; siRNA, small interference RNA.

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Androgen receptor and cellular survival in prostate cancer

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Introduction

Prostate cancer is the most common cancer diagnosed after skin cancers and the second leading cause of cancer death in the US. It is estimated that there will be about 232,090 new cases and 30,350 men will die because of this disease in 2005 (1). Since it is a severe health threat to men, extensive studies have been conducted on this disease in recent years and the research advances were summarized by numerous excellent review articles in all aspects including general mechanism (2-9), cancer genetics and epidemiology (10-18), androgen receptor (AR) biology (19-40), AR coregulators (41-42), AR cross-talk with other signal pathways (43-47), antiandrogen

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and novel therapies (48-60), as well as new biomarkers for prognosis and diagnosis (61-63). In this review, thus, we will only discuss the survival pathways related to the AR signaling in prostate cancers.

Androgen receptor is the key molecule for prostate cancer progression

Since the seminal work of Huggins and Hodges in 1941 (64), it has been widely accepted that androgens play a critical role not only in the physiological development of the prostate but also in the genesis of prostate cancer. Currently, androgen ablation is the major therapeutic approach for advanced disease. However, most patients treated by androgen ablation ultimately relapse to more aggressive androgen-independent or so-called hormone-refractory (because it is resistant to hormone ablation therapy) prostate cancers. The etiology of hormone-refractory progression may have various molecular causes, but the AR is expressed and its function is maintained in each scenario (reviewed in Ref. 19-40), suggesting that androgen-independent AR signaling is involved. Since the original cloning of the AR gene in 1988 (65), the role of AR in prostate development and cancer progression has been extensively studied. Especially, the essential role of the AR for hormone-refractory progression was demonstrated recently (66-69).

The first report showing a direct connection between the AR and cell growth in androgen-independent prostate cancer cells under androgen deprivation condition was published in 2002 by Dr Tindall's group (66). In a cell-based experiment, disruption of AR activity using an AR antibody to block its function or AR-specific ribosome to suppress its protein translation caused a significant growth inhibition either in androgen-dependent or -independent prostate cancer cells that harbor a native *AR* gene. However, there was no significant inhibition of cell growth upon exposure of prostate cancer DU145 cells that are null of *AR* gene to those procedures. This initial observation was supported by a study using xenograft model of prostate cancer (67). The transition from androgen-dependent to -independent stage was established using human prostate cancer LAPC-9 xenograft line in severe combined immunodeficiency (SCID) mice. Then, AR function was determined using androgen responsive reporter gene assay and AR-DNA binding (chromatin immunoprecipitation, ChIP) analysis. In androgen-dependent stage, the AR localized to the nucleus and bound to the endogenous prostate-specific antigen (PSA, a typical androgen response gene) enhancer. Following castration, the AR disassociated from the chromatin and moved out of the nucleus. However, in recurrent tumors (androgen-independent stage), the AR re-entered into the nucleus and rebound to PSA enhancer region on the chromatin. Reporter assays also showed that the AR was re-activated upon

tumor re-occurrence. This study clearly demonstrated that the AR is fully functional in recurrent androgen-independent tumor after androgen withdrawal. Later on, the critical role of the AR molecule was further determined in a more comprehensive analysis (68). Firstly, AR expression at both transcriptional and translational levels was determined in seven xenograft models, and the results showed that the most profound alteration after castration was a dramatic increase of AR mRNA and protein levels. Secondly, the causative role of increased AR expression on androgen-independent transition was tested. As expected, enhanced AR expression significantly promoted cell growth under androgen deprivation condition in cell culture, castration condition in xenograft mice model, or even in the presence of anti-androgens. Thirdly, the mechanism involved in AR-mediated progression was studied, and the data indicated that the AR was still ligand dependent for its transactivation and the genomic effect is necessary for AR-mediated androgen-independent transition. Taken together, these studies established that the AR is playing a critical role in prostate cancer progression.

In considering its functional role of the AR in prostate cancer progression, targeting the AR but not the androgenic hormone becomes more realistic in terms of therapeutic strategy. Most recently, in an effort to develop novel therapies for prostate cancer, we used the RNA interference technique to silence the AR expression in prostate cancer cells. Several small-interfering RNA (siRNA) duplexes were created and tested for knocking down AR protein. In addition to cell arrest as reported previously (68), surprisingly, a massive apoptotic cell death was observed after siRNA transfection in AR-native prostate cancer cells regardless of their androgen dependency (69). This is the first report showing a direct link between AR molecule and cell death in prostate cancer cells, which further confirmed previous reports that the AR is the key molecule in cellular survival of prostate cancer cells.

Androgen receptor and its expression

The androgenic hormones transmit their regulatory signals to the nucleus through the cognate androgen receptor (AR), which is a ligand-activated transcription factor and a member of the nuclear receptor superfamily. The AR is a member of nuclear receptor superfamily produced from a single-copy 90-kilobase pair (kbp) gene located on the X chromosome at Xq11-12 (65). Without androgen stimulation, the AR is inactive and sequestered in the cytoplasm as a multiprotein complex with heat shock proteins and immunophilins. After the receptor binds androgens, the AR is disassociated from the multiprotein complex, and it undergoes a conformational change, homodimerization such that a nuclear localization signal (NLS) present in the receptor structure is exposed, allowing import of the ligand bound AR to the nucleus. Binding of importins to the exposed NLS is needed for this translocation

process. The transcriptionally active AR accumulates within a subnuclear compartment that is microscopically visualized as nuclear foci (70).

During the development and progression of prostate cancer, multiple alterations occur in the AR molecule and its signaling pathway, including genetic mutations, gene amplification, protein overexpression and functional deregulations. In clinical settings, almost all the prostate cancers express the AR except for the small cell carcinomas, and no correlation was found between the AR expressing levels and clinical outcome. However, compared to primary cancers, hormone-refractory cancers display an increased AR expression at both mRNA and protein levels (20, 72-73). In a recent study using matched paired hormone-sensitive and resistant tumors (74), the copy numbers of AR locus on X chromosome was found to increase in 20% (10 out of 49) cases while only 2% (1 out of 48) of primary tumors displayed multiple AR copies. Consistently, tumors with higher copies of the AR gene showed significant high level of AR protein level in those matched cases. In addition, overexpression of AR protein was also detected in 35% of cases lacking AR gene amplification, indicating that alternative mechanisms exist for the increase of AR protein levels (i.e. decrease in degradation or increase in stabilization). In deed, enhanced stabilization of AR protein was found to be associated with the development of hormone-refractory tumor in a mouse xenograft model (75), resulting in hypersensitivity to castrated levels of androgens. In a well-designed PCR-based study (76), AR expression at the mRNA level was determined based on the cell type using cell type-specific RNA references. Compared to normal prostate tissues and localized primary tumors, epithelial levels of AR mRNA were significantly higher in hormone-refractory tumors. However, there is no significant difference in AR levels between normal tissues and primary tumors. Another group also reported similar results based on a cDNA microarray analysis using patient cancer tissues (77). Since expression of some AR-regulated genes (i.e. PSA and PAP) were not up-regulated in those cases with AR amplification (74), it is not clear if the increased AR expression levels reflect the likelihood of hormone refractory transition. Otherwise, increased AR expression might be involved in other signaling pathways that are responsible for hormone refractory progression of the disease.

Androgen-stimulated AR transactivation

Currently, the regulation of ligand-induced AR transactivation in a target cell is only partially understood, and most of the proposed mechanisms for AR transactivation are based on the work for the GR, a glucocorticoid receptor, which is a close-related member in structure to the AR (21). Classically, in response to androgen stimulation (binding), cytoplasmic localized AR is dissociated from the chaperon heat shock protein 70/90, phosphorylated and

then translocated into nuclear compartment for its genomic actions after interaction with chromatin although a DNA binding-independent mechanism for AR transactivation was also reported (78), and a recent review article exclusively summarized this part of current understanding (79).

In a tempt to dissect the mechanisms involved in androgen-induced AR transactivation, we recently demonstrated that androgen-induced AR transactivation involves two major cellular signaling molecules, phosphoinositide 3-OH kinase (PI 3-kinase, PI3K) and glycogen synthesis kinase-3β (GSK-3β) (80-81), which is consistent with previous reports (82-83). Inhibition of their function using specific inhibitors or using small interference RNA approach to knock down GSK-3β protein expression resulted in a suppression of androgen-mediated gene reporters or endogenous gene expression but not nuclear translocation of the receptor. Androgen treatment increased GSK-3β tyrosine phosphorylation that is associated with its enzymatic activity, while pretreatment with PI3K inhibitor abolished androgen-induced GSK-3β activation. The mechanism involved in androgen-mediated PI3K activation is under further investigation; however, a possible involvement of guanine nucleotide-binding proteins (G-protein) has emerged (84-86). Androgen-induced AR-responsive reporter activity was enhanced by overexpression of a Rho guanine nucleotide dissociation inhibitor (RDIα) (84-85), which is a negative regulator of small G-protein RhoA. Previous reports showed that RhoA can down-regulate PI3K activity (87-88). In addition, the trimeric G-protein subunits alpha-s and -q were reported to enhance AR transactivation when only a trace level of androgen is available (86), suggests that G-proteins are providing both positive and negative signals to androgen-mediated AR transactivation (89-90).

Androgen-regulated genes

As a transcription factor, ligand-activated AR interacts with chromatin at the androgen response element (ARE) in a given target gene promoter/enhancer region and regulates gene expression in cooperation with the general transcription machinery. The molecular communication between the AR and transcription machinery was recently reviewed elsewhere (91), thus we will focus on the target gene groups regulated by the AR.

As discussed above, androgens, acting via the AR, are central to prostate development and carcinogenesis, and understanding AR-regulated genes at the molecular basis in prostate cancer will potentially improve patient care and outcome. Using a high-through put genomic scale analysis, AR-regulated genes in prostate cancer, cell lines or patient tissues, were determined by several research teams (77, 92-94), and the listed genes were verified at the transcription level by RT-PCR or at protein level by Western blot and

immunostaining. In general, there are five groups of genes stimulated by androgen treatment, including cell proliferation, differentiation, apoptosis, metabolism, and secretory activity (79, 92-94). All prostate-derived AR-positive cancer cell lines, regardless of their androgen-dependency or AR mutation, displayed similar pattern of gene expression after androgen stimulation, indicating conservation of specific androgen responsiveness (92). The majority of androgen-stimulated genes are those involved in prostatic secretory fluid, which is the main function of the prostate gland (92). In two other reports, changes of AR-regulated genes were determined after androgen ablation or AR elimination (77, 95). In human prostate cancer cell LNCaP, androgen ablation or AR elimination by antisense approach resulted in several concordances in terms of gene expression alteration, including genes related to proliferative and cell cycle machinery, and fatty acid metabolism. However, some differences were also revealed. For example, AR elimination led to profound downregulation of insulin-like growth factor binding protein 2 (IGFBP-2) and the phosphatidylinositol-4-phosphatase 5-kinase type I alpha (PIP5KIA), while androgen blockage induced a dramatic change in the expression of the prostate overexpressed gene 1 and the S100 calcium binding protein P (S100P). These results suggest that a functional difference exits between androgen ablation and AR elimination (95).

Progressing to hormone refractory is currently a critical issue for curing prostate cancers, and understanding the genes that abnormally expressed in those late stage tumors will provide potential therapeutic targets. A recent study performed a genome-wide analysis of human prostate cancers during androgen ablation therapy (77), and they found that the hormone-refractory tumors showed a similar overall expression profiles as the untreated primary tumors in a hierarchical clustering algorithm analysis. These results indicate that a reversal event occurred when the tumor progresses to hormone refractory stage. In this process the effect of androgen ablation on prostate cancer cells disappeared and the AR signaling is reactivated (77, 94). However, a unique set of genes was also identified from those refractory tumors, in which increased AR expression is the most profound one that may contribute to the reactivation of the AR signal pathway. Another intriguing finding is that several genes involved in steroid precursor synthesis also increased in hormone refractory tumors.

Androgen receptor-mediated survival pathways

Apoptosis, or programmed cell death is a well-conserved process whose basic tenets remain common to all metazoans (96-97). It is usually controlled by two major execution pathways: the death receptor pathway or so-called extrinsic pathway; and the mitochondrial or intrinsic pathway. The extrinsic pathway is activated by binding of ligand to the death receptors located at the

plasma membrane such as the receptors for Tumor Necrosis Factor α (TNF-α), Fas ligand and TNF-α-related apoptosis-inducing ligand (TRAIL). Intracellular organelles, like mitochondria, are key participants in apoptosis. The main aspects of mitochondrial involvement in apoptotic process include two critical events, the release of mitochondrial proteins such as cytochrome c and the onset of multiple parameters of mitochondrial dysfunction such as loss of membrane potential.

In addition to the development in embryonic stage, the proliferation and differentiation in puberty and the physiological function in adulthood of the prostate gland, androgens are also critical for survival to the prostatic epithelial cell throughout its entire life. It is well demonstrated that the prostate gland responding to castration in rodent models or human cell lines responding to androgen deprivation or antiandrogens have shown a rapid apoptosis of the prostate epithelial cells, resulting in an extensive glandular regression (98-100). There is now evidence for a critical role of AR-mediated signaling pathway in cellular survival, although other AR bypassing pathways including castration-reduced blood flow are also emerging (101-102). The castration effects on blood flow to the prostate gland seem to be related to vascular degeneration associated with apoptosis of a subset of prostate endothelial cells (103). However we will mainly discuss the AR-mediated signaling pathways involved in cellular survival of prostate cancer. Currently only a few downstream targets involved in this pathway are identified.

Functioning as a nuclear transcription factor, the AR mainly exerts its effect through regulation of gene expression or so-called genomic effect although a DNA binding-independent mechanism was reported for AR transactivation by triggering of AR coactivators in some circumstances (78). Consistent with this notion, introduction of synthetic “decoy” androgen response element (ARE) oligonucleotides induced cell death in human prostate cancer LNCaP cells (104). On the other hand, a non-genomic effect of the AR through a protein-protein interaction mechanism on cell survival was also reported recently (105). Based on this knowledge AR-mediated survival pathways might be summarized in two aspects: genomic and non-genomic effects.

Cell cycle progression and cellular proliferation are controlled by cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CDKIs). CDKs are sequentially activated upon association with their partner cyclins. There are two checkpoints in each cell cycle: the G1/S checkpoint controls initiation and completion of DNA replication, and the G2/M checkpoint controls mitosis and cell division (106-107). Functional regulation of cyclin/CDK complexes by its inhibitors (CDKIs) is critical for cell cycle regulation at or before the checkpoints. There are two groups of CDKIs that control the checkpoints. The first one comprises $p16^{INK4a}$ and $p15^{INK4b}$, as well as p18 and p19 (106). Each

of these genes encodes a protein that specifically inhibits CDK4 and CDK6. Another group of CDKIs consists of $p21^{WAF1/CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$, which has considerable sequence homology and can inhibit all CDKs (106). In addition to G1 phase cyclins (cyclin A and B1) and CDKs such as CDK1, CDK2, CDK4 (95-96), CDKI $p16^{INF4}$ and $p21^{WAF1/CIP1}$ are also regulated by the AR in prostate cancer cells (108-112).

The CDK inhibitor $p21^{WAF1/CIP1}$ is a multi-function protein involving in cell proliferation, DNA repair and survival (106, 113-114). Although there are some evidences from the clinical studies and mouse xenograft models that $p21^{WAF1/CIP1}$ is involved in prostate cancer progression (116-122), its actual role in this process is still controversial. In experimental situations, opposite findings were reported (110-111, 114, 123). Earlier studies showed that $p21^{WAF1/CIP1}$ expression is stimulated by androgen treatment at a transcriptional level and an androgen responsive element (ARE) was identified in the promoter region of *p21* gene. Meanwhile, the basal level of $p21^{WAF1/CIP1}$ protein increased and was further enhanced by androgen stimulation in androgen-independent LNCaP sublines compared to its parental cell line (110-111). The authors stated that AR-mediated $p21^{WAF1/CIP1}$ expression might participate in androgen-induced antiapoptotic effect. On the other hand, some other reports provided evidences that p21 is inversely correlated with cell survival during prostate cancer progression (114, 123). Overexpression of $p21^{WAF1/CIP1}$ in prostate cancer cells not only resulted in cell arrest but also caused a significant apoptotic cell death (114). During the transition from androgen dependent to independent stage, AR expression increases but $p21^{WAF1/CIP1}$ level decreases in parallel in LNCaP cells. Meanwhile, a functional link between the AR and $p21^{WAF1/CIP1}$ expression was established by the fact that androgen treatment reduced largely the $p21^{WAF1/CIP1}$ protein level and inhibited $p21^{WAF1/CIP1}$ promoter activity via the putative ARE motif located in the promoter region. Furthermore, elimination of AR protein by an antisense oligonucleotide enhanced $p21^{WAF1/CIP1}$ promoter activity and increased $p21^{WAF1/CIP1}$ protein level in androgen-independent LNCaP cells (123). These evidences indicate $p21^{WAF1/CIP1}$ as an inducer of apoptotic cell death in prostate cancer cells, which is totally controversial with the aforementioned conclusion and is just exactly like the role of $p21^{WAF1/CIP1}$ itself in cell survival or death depending on cellular condition and stimuli (123-124).

The second downstream target involved in AR-mediated survival is the forkhead transcription factors in rhabdomyosarcoma (FKHR) family member FOXO1 (105, 125). The FKHR family proteins play an important role in many cellular processes including cell proliferation through regulation of CDK inhibitor $p27^{kip1}$ and D-type cyclones (126-127), cyclin B and polo-like kinase (128), as well as cyclin G2, EXT1 and Wip1 (129). This group of proteins is also involved in cell survival by up-regulating pro-apoptotic proteins such as Fas ligand, the insulin-like growth factor-binding protein 1 (IGFBP-1) and

Bcl-2 family protein Bim (129-135), as well as interacting with peroxisome proliferative activated receptor-gamma co-activator 1 (PGC-1, a transcription co-activator). In deed, overexpression of active FKHR leads to apoptotic cell death in human prostate cancer LNCaP cells (131, 137).

Two different mechanisms were proposed for AR-mediated survival through inhibition of FKHR action, AR interaction with FKHR (105) and AR-mediated FKHR degradation (125). In AR-null prostate cancer DU145 cells, overexpressed AR inhibited FKHR-mediated gene expression and cell death, which was not due to a competition of AR with FKHR for transcription co-factors but due to a complex formation between AR and FKHR. This interaction was also confirmed in an *in vitro* protein binding assay (105). However, AR-mediated enhancement of FKHR degradation was reported in LNCaP cells that express a native mutant *AR* gene. When the cells were treated for a rather long period of time (48-72 hours) with androgen, a cleavage fragment (p60) of FKHR protein was detected while a 24-hr treatment of androgen did not cause any changes of FKHR protein (105). Proteomic approach confirmed that the fragment is a c-terminal truncation of FKHR protein, which was cleaved by lysosomal acidic cysteine protease. This truncated form of FKHR in turn inhibits the function of the intact form of FKHR (125). Due to the heterogeneous feature of prostate cancers (138), both mechanisms for AR-mediated FKHR inhibition needs further verification.

The Bcl-2 family proteins are critical regulators that directly control the mitochondria function and consist of both pro- and anti-apoptotic members (reviewed in Ref. 139). Bax, Bak, and Bok are proapoptotic members, as are the BH3-domain only members such as Bad, Bik, Bim, and Bid. Antiapoptotic members include Bcl-2 and Bcl-x_L, Bcl-w, Mcl-1, and etc. It is believed that the relative levels of pro- and anti-apoptotic members are the key determinants in the regulation of cell death and survival. Bcl-2 protein was once reported to be elevated by androgen treatment in LNCaP cells in an earlier study (140), which was not confirmed by later reports (141-143). In contrast, Bcl-2 expression was found to increase after androgen ablation therapy, serving as a potential mechanism for androgen-independent progression of prostate cancer (reviewed in Ref. 144).

As another major anti-apoptotic member of Bcl-2 family, Bcl-x_L was recently identified by our group as a downstream effecter of AR-mediated cell survival pathway (69). As described earlier, in parallel to AR silencing and cell death, Bcl-x_L was the only member of Bcl-2 family proteins that was down-regulated. Consistently, enforced overexpression of Bcl-x_L partially protected cell death induced by the AR siRNA. More convincingly, our unpublished data showed that androgen treatment increased Bcl-xL expression at the transcription level, and the AR was found to bind to a putative androgen response element in the promoter region of human *bcl-x* gene (Fig 1). Taken

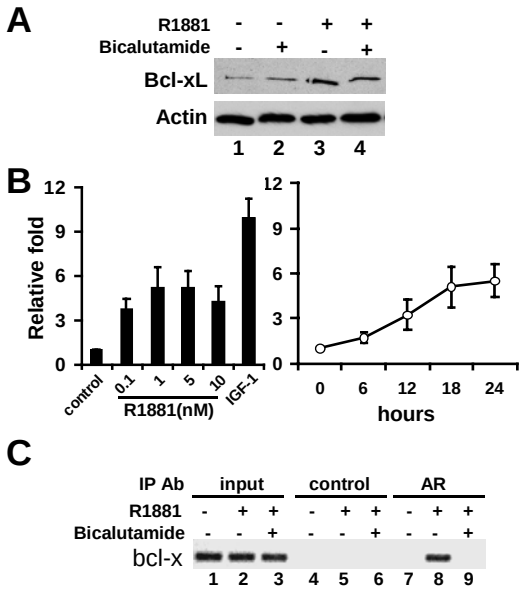

Figure 1. Androgen stimulates Bcl-xL gene expression *via* an AR-dependent mechanism. (A) After serum starvation for 24 h, LNCaP cells were treated with R1881 (1.0 nM) in the present or absent of bicalutamide (10 μM) for another 24 h. Cells were harvested and Bcl-xL protein level was determined by Western blot, and Actin blot served as loading control. **(B)** LNCaP cells were co-transfected with a luciferase reporter construct pBcl-xL-LUC together with an internal control reporter construct pCMV-SEAP overnight and then were serum-starved for 24 h. The solvent ethanol (control), R1881 in different doses as indicated or IGF-1 (10 ng/ml) alone was added once in the culture media containing 2% cFBS for another 24 h (left-half panel) or for several different time-points as indicated (right-half panel). Luciferase or SEAP activities were measured, and the luciferase activity was presented as fold induction against control sample after normalized with protein content and SEAP activity. **(C)** ChIP assay. LNCaP cells were serum-starved for 24 h and then untreated or treated with R1881 (1.0 nM) for 18 h in the presence or absence of the antiandrogen bicalutamide (10 μM). Binding of AR to the bcl-x promoter was determined with the ChIP assay as described in the text (lanes 7-9). As controls, sample lysates were also incubated with a normal rabbit serum IgG (lanes 4 and 6). Lanes 1 and 3 represent input signals obtained from 1% input chromatin. IP Ab, immunoprecipitation antibody.

together, our work demonstrated that Bcl-xL is a downstream target involved in AR-mediated cell survival in prostate cancer cells.

The fourth downstream target in AR-mediated survival is the c-FLIP (cellular FLICE-like inhibitory protein). FLICE [FADD (Fas-associated death

domain)-like interleukin-1β-converting enzyme] is one of the former names for caspase-8, which is one of the initial caspases (Caspase-2, -8, -10) involved in death receptor-mediated apoptosis (145-146). There are two variants of c-FLIP, short and long isoforms and most of the studies were dealt with the long form (c-$FLIP_L$) (146). Like caspase-8, c-$FLIP_L$ contains N-terminal tandem death effecter domains (DEDs), but its caspase domain is enzymically inactive that differs from caspases-8. In contrast, c-$FLIP_S$ only has two N-terminal DEDs that are very similar to the prodomains of caspase-8. Since the structural similarity, c-FLIPL is believed as an ideal inhibitor of death receptor-mediated apoptosis pathway. This concept was supported by data such that c-FLIP knockout mouse embryonic fibroblasts (MEFs) are more sensitive to death-receptor-induced apoptosis (147).

Almost all prostate-derived cancer cell lines are resistant to death receptor-mediated apoptosis compared to leukemia cells (148); however, this resistance can be reversed by additional reagents, for example, glycogen synthesis kinase-3β (GSK-3β) inhibitors as seen in our recent publication (149). In attempt to identify any gene alterations during castration-induced apoptosis in rat prostate, mRNA levels of genes encoding components of death receptor-mediated apoptosis pathway were analyzed using a quantitative real-time RT-PCR technique (150). The mRNA level for c-FLIP was the only one that reduced dramatically within the first 12 hours after castration, while the mRNA level of its interaction partner caspases-8 was not changed. Most interestingly, androgen administration to castrated animals restored the c-FLIP expression to normal levels, indicating an androgen-dependent regulation. Consistently, another report showed that androgen treatment activates c-FLIP promoter activity and gene expression in prostate cancer cells; and the AR is indeed recruited to the promoter region of human c-FLIP gene and several putative androgen response elements were identified (151). Overexpression of c-FLIP in prostate cancer cells confers its resistance to death receptor-induced apoptosis and promotes tumor progression in a mouse xenograft model (151-152). These reports are also somewhat in agreement to our findings that prostate cancer cells can be sensitized to death-receptor-mediated apoptosis by suppression of GSK-3β activity (149); meanwhile, GSK-3β activity is required for androgen-induced AR transactivation (81) although c-FLIP expression was not determined in our work.

Androgen receptor-related survival pathways

In recent years, androgen receptor-mediated non-genomic effect on multiple signaling pathways has emerged as an alternative mechanism for androgen action (153). In androgen-sensitive epithelial cells including prostate cancer LNCaP cells, the AR interacts and activates a major cellular signaling

cascade, the phosphoinositide 3-OH kinase (PI 3-kinase)-protein kinase B (also called Akt) signaling pathway (154-157). It has been very clear that PI-3k-Akt pathway plays an important role in providing cells with a survival signal that allows them to withstand apoptotic stimuli (reviewed recently in ref. 158-159). Upon activation, PI3K produces more lipid products such as phosphatidylinositol 3, 4, 5-trisphosphate and phosphatidylinositol 3, 4-bisphosphate on the inner surface of the plasma membrane, which is responsible for Akt activation (160-162). In contrast, a dual phosphatase PTEN (phosphatase and tensin homologue deleted from chromosome 10) dephosphorylates lipid products produced by the PI3K (163), resulting in inactivation of PI3K-Akt cascade. Somatic mutation or deletion of PTEN is a rather common event in solid tumors including prostate cancer (164-165), causing elevated levels of PI 3-kinase products and overactive Akt cascade. As a result those tumor cells are relatively resistant to apoptosis (166).

In early studies, the PI3K-Akt cascade was demonstrated as a dominant survival signal in a cell culture or mouse xenograft model based on LNCaP line harboring a mutant PTEN gene (167-169). Inhibition of the PI3K-Akt pathway but not other signal pathways caused a rapid apoptotic response, which was attenuated by androgen addition. This observation was confirmed by other reports (170-171). During the androgen independent transition after removal of androgens from the culture, LNCaP cells showed an increased level of PI3K-Akt cascade activity, indicating a compensational change had emerged. Increased Akt activity, enhanced phosphorylation of Akt downstream targets including pro-apoptotic Bcl-2 protein Bad, and diminished expression of CDK inhibitor $p27^{kip1}$ were observed in androgen-independent tumors, indicating their association with prostate cancer progression (168-169, 171). Although the mechanism responsible for androgen protection of PI3K inhibitor-induced apoptosis remains largely unknown, a possible role of Bcl-x_L was suggested (172). Ectopic overexpression of Bcl-x_L protein protected LNCaP cells from PI3K inhibitor-induced cell death, indicating Bcl-x_L is mediating a PI3K/Akt-independent survival cascade. In our recent study we demonstrated that Bcl-x_L is a downstream target of the AR survival pathway (69), which provides a possible mechanism in androgen protection against PI3K inhibition-induced cell death.

Targeting the AR as a novel strategy in prostate cancer therapy

Currently androgen-independent (or so-called hormonal refractory) phenotype is the major obstacle to curing prostate cancers. Androgen ablation by chemical or surgical approaches and AR blockage by anti-androgens have been shown to fail in controlling the disease (173). In certain circumstance, removal

of anti-androgens has been reported to result in regression of the disease (termed as androgen withdrawal syndrome, reviewed in ref. 51). Thus novel therapeutic strategies that target the disease at a molecular level are desirable to prevent or disrupt its progression.

As discussed above, the AR has been demonstrated as a critical molecule in prostate cancers, and then shifting the therapeutic target from androgens to the AR itself will be more realistic and efficient in terms of erasing its action on disease progression (174). A group from Austria reported recently that using an antisense oligonucleotide approach against the AR resulted in a significant inhibition of cell proliferation *in vitro* (175) and tumor growth *in vivo* (176). Among various oligonucleotides with different sizes and targeting segments on the AR sequence, a 15-base oligonucleotide targeting the CAG repeats was identified to be most effective in suppressing AR protein level in an LNCaP cell-based *in vitro* assay. Treatment of the cells with the particular oligonucleotides for 24 hours caused a more than 90% reduction of AR expression. Cell proliferation and PSA secretion were significantly inhibited. A similar effect was also observed in LNCaP-derived subline that has an androgen-independent phenotype. This antisense oligonucleotide was also used in a mouse xenograft model derived from LNCaP cells. Surgical implantation of a diffusion pump containing the oligonucleotides near the xenograft for 7 weeks resulted in around 40% reduction in tumor weight compared to control groups. However, no significant cell death was observed after antisense oligonucleotides treatment compared to control group although the cell proliferation maker Ki67 was found to be correlated with tumor size. These pioneer works pointed out that targeting the AR might be a permissive approach for curing prostate cancers although more improvements are needed to enhance its efficiency.

Targeting the AR for prostate cancer treatment is also the major focus of our group. Using a more efficient and gene-specific silencing approach, RNA interference, we demonstrated that silencing AR expression in native AR-positive prostate cancer cells led to a massive cell death (69). Using a computer-based design tool (*OligoEngine*™, www.oligoengine.com), we synthesized several small interfering RNA (siRNA) duplexes and tested them on different prostate cancer cell lines. Of 34 siRNA duplexes, we identified 4 duplexes that efficiently knocked down AR expression in mRNA and protein levels by 3-4 days after transfection. To our surprise, in addition to cell arrested as reported by others using AR siRNA (68, 177), a massive apoptotic cell death was observed when the siRNA-transfected cells were kept in a hormone-free condition for up to 4 days. Only a tract percentage of cells could survive after 12 days under the condition. These phenomena occurred in all AR-positive prostate cancer cells regardless of their androgen dependency. However, no cell death was induced in AR-null prostate cancer cells or such

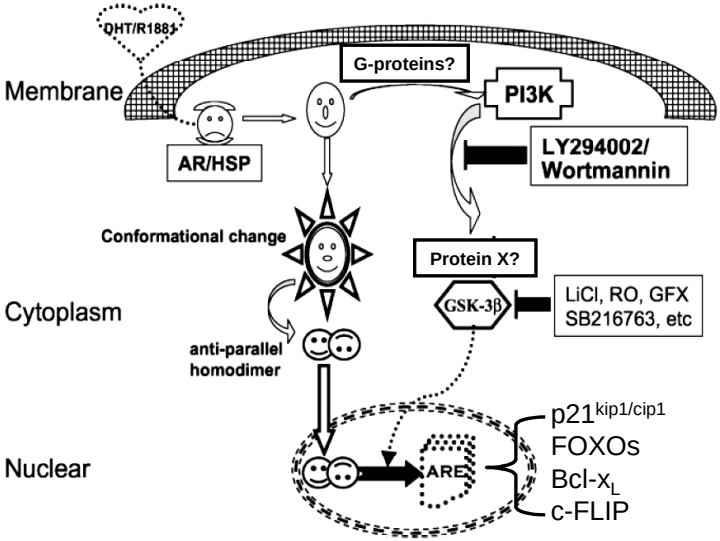

Figure 2. Schematic illustration of the proposed mechanism of AR transactivation in prostate cancer. Upon androgen binding, the AR is dissociated from the chaperon heat shock proteins (HSP) and then translocated into the nuclear as an antiparallel homodimer after a conformational change. In addition, androgen treatment also results in PI3K activation, possibly via G-proteins, which in turn leads to GSK-3β tyrosine phosphorylation through unknown mechanism. Finally, activated GSK-3β regulates the assembly of AR-mediated transcriptional complex in the nuclear.

cells that were even reconstituted with an exogenous *AR* gene, indicating the AR-null cells escaped already from AR control for survival. In addition, no cell death was found in breast cancer cells although they are AR-positive, suggesting a cell-specificity for AR-mediated survival. These results suggested that targeting the AR with the siRNA approach is a powerful therapeutic measurement to treat prostate cancers at any stage as long as they express the AR.

Conclusion

Prostate cancer is a major health threat to Western men, and currently no efficient cure exists when it becomes resistance to androgen ablation therapy. The AR gene as well as its protein product is the only one constantly and profoundly upregulated during progression of the disease. In prostate epithelium or its cancerous compartment, the AR controls most of the aspects of cell fate including proliferation, differentiation, secretion and survival.

During the development and progression of prostate cancer, the AR is always a critical molecule. Overexpressed AR promotes cell growth under a trace level of androgen *via* its genomic effects. Under castration condition, the AR is still active in cancer cells after reoccurrence *in vivo* but the mechanisms are not fully clear. Blocking its function using a specific antibody or reducing its expression using a gene-specific ribosome or antisense oligonucleotides led to growth inhibition both *in vitro* and *in vivo*. Silencing the AR gene using a more efficient approach, RNA interference, could induce a massive cell death after a short period of growth inhibition. AR siRNA-induced cell death was observed in both androgen-dependent and -independent prostate cancer cell lines but not in AR-null prostate cancer cells or none-prostate cells. The siRNA-based approach might be a permissive measurement for curing prostate cancers although further testing in a mouse xenograft model is desirable.

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CHAPTER 5

Nano-Scaled Techniques in Prostate Cancer Management: From Basic Research to Clinic Settings

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CONTENTS

INTRODUCTION

Nano-scaled technique, or so-called nanotechnology, is defined when the size of a man-made device or an essential component is within a range of 1–100 nm in at least one dimension [1]. Currently, there are three major implications of nanotechnology for cancer management, including extremely sensitive nanobiosensors for cancer marker detection, contrast nanoparticles for molecular imaging, and injectable non-viral vectors for targeted drug delivery. In this chapter, we will provide an up-to-date overview of the implications of nanotechnologies in prostate cancers.

1. NANO-BIOSENSOR DETECTION OF PROSTATE-SPECIFIC ANTIGEN

In the US, one out of six men will be diagnosed with prostate cancer during his life-time, and more than 20,000 men are diagnosed with prostate cancer every year, which makes prostate cancer the most diagnosed non-skin cancer and the second leading cause of cancer-related death [2].

Currently, prostate-specific antigen (PSA) is the most commonly used biomarker for *ex vivo* detection of prostate cancer. It is a 33-kDa serine protease and belongs to the kallikrein gene family. It was first isolated from human semen in 1973 [3] and then purified from prostate tissue as a prostate-specific protein in 1979 [4]. PSA is produced and secreted into semen by prostate epithelium, and plays a key role in semen liquefaction [5].

As a cancer biomarker, sensitivity, and specificity are the most important criteria. Before PSA was adopted for prostate cancer detection, prostatic acid phosphatase (PAP) was measured to monitor disease progression. Because PAP was not sensitive to localized cancers, it was replaced by PSA in the mid-1980s [6]. Since then, PSA screening has been widely utilized as a test for early detection of prostate cancer. However, recent studies have shown that PSA testing based on current technologies fails to detect a significant

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Table 1. Reports on effectiveness of free-to-total PSA ratio in detection of prostate cancer.

References	Immuno-assay type	Blood sampling and storage	Pt. char-acteristics	No. Pts.	No. pos. result/ neg. result	Age (yrs.)	Area under curve		Free-to-total PSA cutoff	Sensitivity	Specificity	Pos. predictive value	Neg. predictive value
							PSA	Free-to-total PSA					
Kuriyama et al. [91]	Immulyze HS*	Whole blood kept 8 hrs. at 25C and then analyzed	Nonscreened, nonconsec-utive PSA 4.1–10 ng/ml	121		—	0.598	0.745	0.23, 0.16	90, 85	19, 57	—	—
	PSA (free PSA and total PSA)				36/85							—	—
Partin et al. [92]	Hybritech	Eliquated in 3 hrs., stor-age at −70C	Nonscreened, consecutive PSA 4–10 ng/ml	219		Mean 65	—	0.612	0.25	95	20	—	—
	Tandem-R†				31/69								
Trinkler et al. [93]	Immulite* (total PSA and free PSA)	Analyzed on day of collection; storage at 4C	Nonscreened, nonconsec-utive PSA 2.5–10 ng/ml	89	23/66 (BPH)	—	—	0.19	70, 0.33	71, 87	46, 23	87, 28	83
Veltri et al. [94]	Tosoh (total PSA)	Eliquated imme-diately, stor-age at −80C	Nonscreened, nonconsecu-tive PSA 2–20 ng/ml	531	271/255 (BPH)	45–91 (mean 68)	0.579	0.727	0.21–0.25 0.31–0.35	Av. 89 Av. 98	Av. 30 Av. 10	Av. 58 Av. 54	Av. 72 Av. 78
	Immulite (free PSA)												
Basso et al. [95]	Immulite	Storage at −20C	Nonscreened, consecutive	330	112/218 (BPH)	49–90 (mean 69/67)	—	—	0.22	58	95	—	—
Hofer et al. [64]	Tandem-R (free PSA),	—	Nonscreenced, nonconsec-utive PSA 4–10 ng/ml	75	32/52 (BPH)	Mean 68	0.610	0.670	0.14, 0.18, 0.21, 0.25	74, 87, 91, 96	31, 58, 44, 35	—	—
	Tandem-E† (total PSA)											—	—
											—	—	
											—	—	
Vessella et al. [98]	AxSYM‡ (free PSA and total PSA)	Storage at −20C (47%), ana-lyzed at 1 yr., storage at −70C (53%), analyzed at 2 yrs.	Nonscreened, nonconsec-utive PSA 4–10 ng/ml	297	100/197	Mean 65	0.476	0.762	0.10, 0.11, 0.24, 0.26	25, 35, 90, 96	95, 90, 40, 27	—	—
			Nonsuspicious digital rectal examination									—	—
Catalona et al. [63]	Hybritech	Storage at 2–8C max. 24 hrs. or at −70C	Nonscreened, nonconsecu-tive PSA 4–10 ng/ml	773	379/394 (BPH)	50–75 (median 64)	0.540	0.720	0.25	95	20	—	—
Horninger et al. [50]	Delfia	—	Screened, con-secutive PSA 2.5–10 ng/ml	308	58/250	Older than 45	—	—	20%	100	55%	—	—

Source: Reprinted with permission from [7], G. Karazanashvili and P. A. Abrahamsson, *J. Urol.* 169, 445 (2003). © 2003, American Urological Association.
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proportion of cancer patients (i.e., false negatives). Also, the low sensitivity of conventional assays render them incapable of determining early recurrence after surgery. Therefore, the critical need in prostate cancer management is a measurement to simply, rapidly, and sensitively detect PSA level to predict the natural course of an individual tumor in each patient [6].

Currently, PSA assays are based on enzyme-linked immunosorbent assays (ELISA) using various labeling techniques. The accuracy of PSA assays vary directly with total serum PSA, and determining the level of free-form PSA helps distinguish the patients who fall into the "diagnostic gray zone" [7]. Table 1 summarized the reports on effectiveness of free-to-total PSA ratio in detecting prostate cancers. For example, a free-to-total PSA ratio threshold of 0.25 resulted in a specificity of 20% and a sensitivity of 95% for the Hybritech PSA assay system (Hybritech Inc., San Diego, CA). Although a question mark has been put on PSA efficacy as a biomarker for the early detection of prostate cancer [6], some improvements have been made using nanotechniques to increase the sensitivity in PSA assay.

Micro- or nano-cantilevers are designed to detect optical deflection or changed resonant frequencies once a molecular interaction occurs on a device surface [8]. This technique is extremely sensitive in detecting trace levels of biomarkers, for example, PSA. Because of their availability and easy integration, silicon-based cantilevers are the most commonly used biosensors. The first reported use of a nanocantilever for PSA detection was a silicon-based device [9], which was made of silicon nitride (Si_3N_4) with a thin layer of gold coated on one surface and with a length of 200 μm, a thickness of 0.5 μm, and a width of 20 μm. The PSA antibodies were immobilized onto the gold film by means of thiol chemistry. The interaction of PSA with immobilized antibodies on the surface bent the cantilever due to intermolecular repulsion, resulting in a deflection that was detected with a laser-based optical system. The deflection increased and then stabilized to a steady-state value over a period of 3–4 hour. Using this device, a wide range of free PSA levels (0.2–60 μg ml^{-1}) was detected with high specificity. For example, without PSA antibody coating, the cantilever showed a negative deflection for a mixture containing a very high level of free PSA, 60 μg ml^{-1}, and 1.0 mg ml^{-1} BSA. On the other hand, with PSA antibody coating but without free PSA in the mixture of 1.0 mg ml^{-1} of BSA alone, no deflection was observed. This high specificity results solely from the changes of free energy on one cantilever surface but not the other [9].

This work provided a strong evidence for cantilever-based technique as a clinical diagnostic tool in prostate cancers. This technology needs no labeling and can be performed in a single reaction without additional reagents. Also, an array of microcantilevers can be used to screen multiple serum markers in a single step. Although it does not demonstrate a sustained advantage compared to conventional methods at this time, nanocantilevers offer an extraordinary multiplexing capacity for multiple detection of disease-oriented expressing signatures in the future [10].

In addition to cantilever bending, mechanical resonant frequency also changes due to a surface stress. Usually, the shift in the resonant frequency is detected using an external oscillator. Most recently, a group from Korea fabricated a nanocantilever using piezoelectric material and established a quantitative electrical measurement under controlled ambient conditions for label-free detection of PSA in a clinically relevant range [11]. This nanocantilever was made of a multilayer film of Ta/Pt/PZT ($Pb(Zr_{0.52}Ti_{0.48})O_3$)/Pt/$SiO_2$ on a Si_3N_4 supporting layer (Fig. 1(a)). The PZT capacitor was formed with 100-mm-diameter p-doped Si wafers and was covered with 1.2-μm-thick low-stress silicon nitride (Si_3N_4). The bottom electrode was made by sputtering a thin Ta adhension layer and a Pt layer with a thickness of 1500 μm. The PZT films were then deposited with a thickness of 0.5 mm by means of a diol-based sol–gel approach. The thickness of the PZT nanomechanical cantilever is 2.08 mm and can be integrated with electronics such as CMOS for electrical sensing and actuating.

To measure the resonant frequency, a superimposed DC voltage (0.25 V) was used to apply an alternating current (AC) sine wave form of 0.5 V pp (peak-to-peak) to the top electrode to vibrate the cantilever. A 30/150-nm-thick Cr/Au layer was deposited on the Si_3N_4 side of the PZT cantilever with an e-beam evaporator to actively detect protein–protein interaction. After cleaning in a solution of piranha ($H_2O_2 : H_2SO_4 = 1:4$), a monoclonal anti-PSA antibody was immobilized onto the cantilever using calix-crown self-assembled monolayers. This device achieved a high sensitivity at as low as 10 pg ml^{-1} of PSA concentration (Fig. 1(b)). This PZT-based electrical nanocantilever has a few advantages over the silicon-based device. It acquires electrical signal without using a costly optical apparatus and

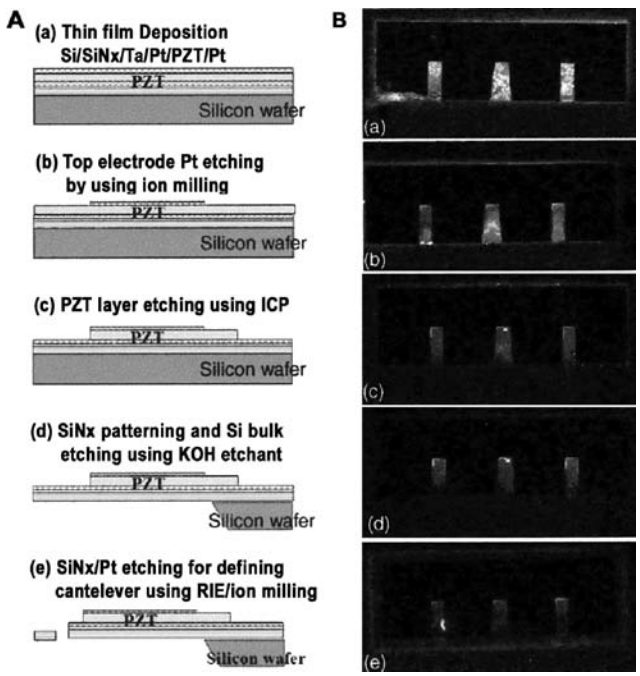

Figure 1. (A) The fabrication flowchart for self-sensing PZT nanomechanical cantilever. (B) The fluorescence scanner images as a function of the interacted PSA antigen concentrations with (a) 100 ng ml^{-1}, (b) 10 ng ml^{-1}, (c) 1.0 ng ml^{-1}, and 0.1 ng ml^{-1}. As a negative control, BSA was used at 1 mg ml^{-1} in (e). Reprinted with permission from [11], J. H. Lee et al., *Biosens. Bioelectron.* 20, 2157 (2005). © 2005, Elsevier Ltd.

external oscillators, and the post-adsorption resonance frequency remains unchanged and the adsorbed agents can be detected repeatedly.

The polymerase chain reaction (PCR) is a powerful tool for detecting trace levels of DNA targets [12]. Recent developments have extended this technique into proteomics for the detection of protein targets, by tagging an antibody with oligonucleotide markers that can be subsequently amplified with PCR for measurement, a technique often referred as immuno-PCR [13]. Using this scheme, a nanoparticle-based bio-barcode sandwich system was established; its sensitivity for detecting PSA is at low attomolar concentrations [14]. As illustrated in Figure 2, this bio-barcode system consists of two types of probes, magnetic microparticles (MMPs, 1-μm-diameter polyamine particles with magnetic iron oxide cores) conjugated with PSA monoclonal antibodies and gold nanoparticles (GNPs), functionalized with hybridized oligonucleotides plus PSA polyclonal antibodies. The captured target and complexed probes are separated by a magnetic field, and then the oligonucleotide bio-barcodes are released by dehybridization, and amplified for determination of the presence of the protein target. Since the nanoparticles carry a large number of oligonucleotide bio-barcodes per protein target–binding events, plus a PCR-based step to further amplify the signal, PSA was detected at the 3-attomolar level (as shown in Fig. 3), which shows a sensitivity 6 orders of magnitude higher than clinically accepted conventional assays (at 3 pM). This system has several

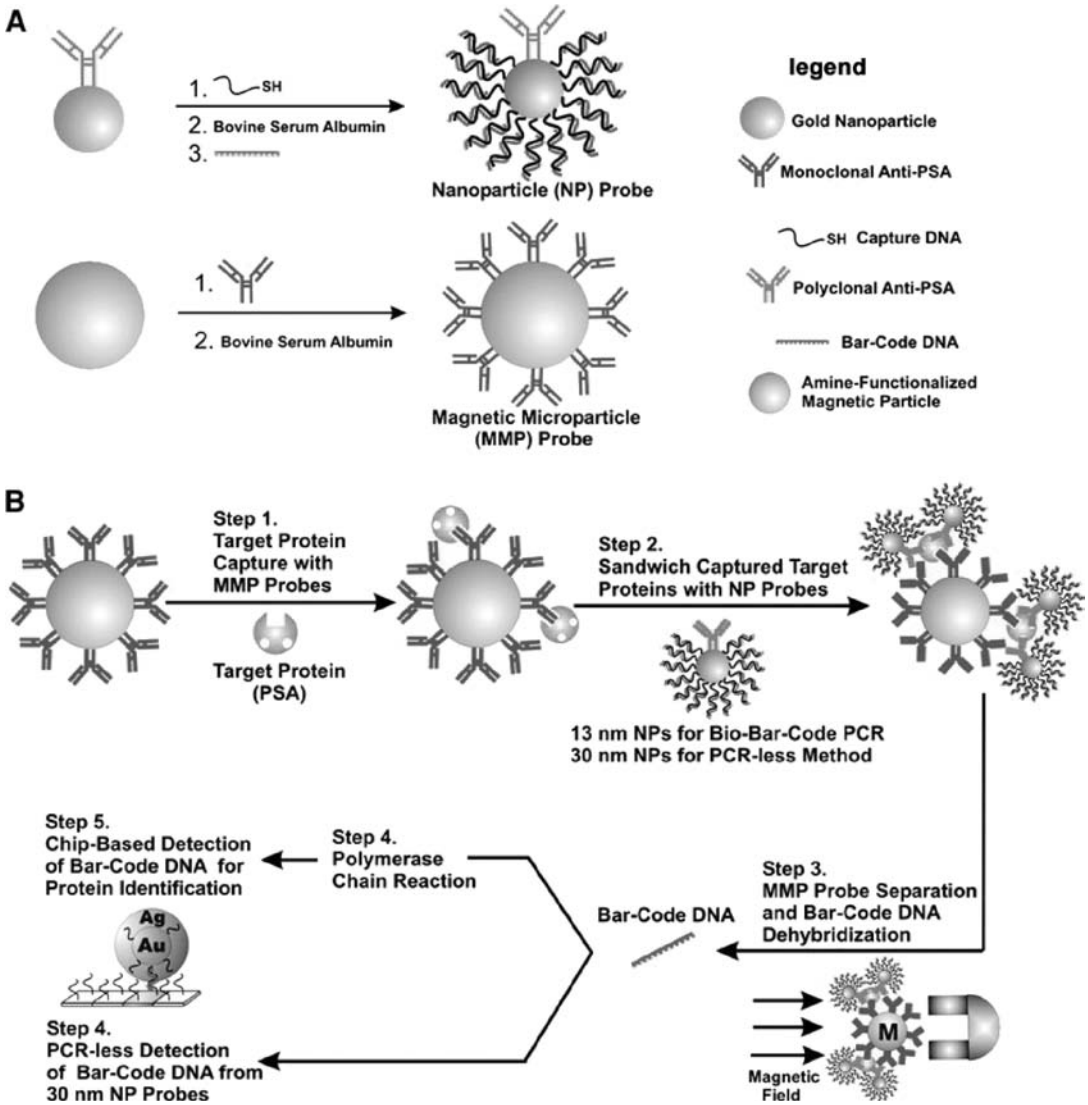

Figure 2. The bio–barcode assay method. (A) Probe design and preparation. (B) PSA detection and barcode DNA amplification and identification. Step 1, in a typical PSA-detection experiment, MMP probes functionalized with mAbs to PSA were mixed with free PSA, the MMP–PSA hybrids were concentrated and resuspended in 0.1 M PBS. The NP probes, which were functionalized with polyclonal Abs to PSA and hybridized barcode DNA strands, were then added to the assay solution. Step 2, the NPs reacted with the PSA were immobilized on the MMPs and provided DNA strands for signal amplification and protein identification. Step 3, after washing, the MMP probes were resuspended in Nanopure water to dehybridize barcode DNA strands from the nanoparticle probe surface. Dehybridized barcode DNA was then separated and collected from the probes with the magnetic separator. Step 4, for barcode DNA amplification. Isolated barcode DNA was amplified, and detected with ethidium bromide. Reprinted with permission from [14], J. M. Nam et al., *Science* 301, 1884 (2003). © 2003, American Association for the Advancement of Science.

advantages over immuno-PCR. Firstly, higher amount of MMPs may be used to enhance the binding kinetics between the detection antibody and target protein. Meanwhile, MMP probes are added homogenously, making this assay more efficient in capturing target protein by increasing the usage of MMP probe, which cannot be done in the heterogeneous assay. Also, this homogeneous mixing protocol is faster than heterogeneous immuno-PCR approaches. Secondly, the use of the NP bio-barcodes increases the sensitivity to a high ratio of PCR-amplifiable DNA to labeling antibody. Thirdly, this system has a potential for multiplexing and simultaneous detection of many protein targets in one solution. Barcode DNA is bound to the NP probe through hybridization at the start of the labeling reaction and liberated for subsequent identification with a simple washing step. Due to the co-conjugation of the antibody and DNA barcode on the same particle, the identification of barcode DNA can be done either by direct detection or by PCR-based protocol, after removing the barcode DNA from the detection assay, which substantially reduces background signal. Finally, this protein detection strategy has great potential for massive multiplexing and the simultaneous detection of many biomarkers in one solution, especially in the PCR-less form. A distinct identifiable barcode can be prepared for nearly every target of interest.

Other immunoassay read-out techniques, such as surface-enhanced Raman scattering (SERS) [15] and surface plasmon resonance (SPR) [16], have been used to detect low level of PSA in human serum samples. A SERS-based system was constructed by coating gold nanoparticles with a monolayer of an intrinsically strong Raman scatterer (Fig. 4). There are three major components in this system: a laser light source, a spectrograph, and a fiberoptic

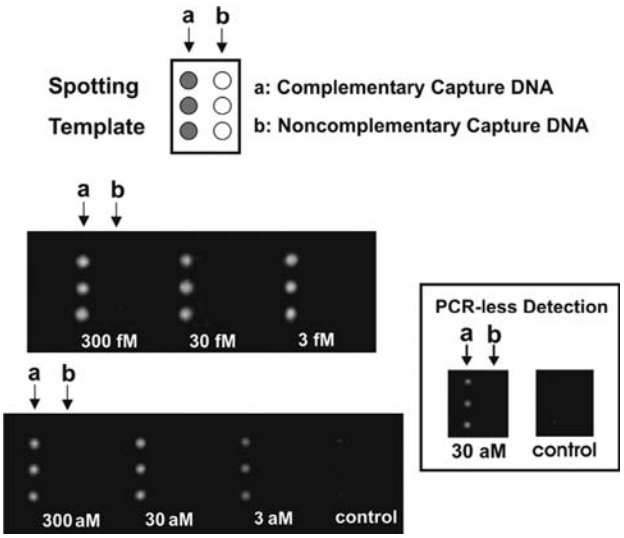

Figure 3. Scanometric detection of PSA-specific barcode DNA. PSA concentration (sample volume of 10 μl) was varied from 300 fM to 3 aM and a negative control sample where no PSA was added (control) is shown. For all seven samples, 2 μl of antidinitrophenyl (10 pM) and 2 μl of β-galactosidase (10 pM) were added as background proteins. Also shown is PCR-less detection of PSA (30 aM and control) with 30 nm NP probes (inset). Reprinted with permission from [14], J. M. Nam et al., *Science* 301, 1884 (2003). © 2003, American Association for the Advancement of Science.

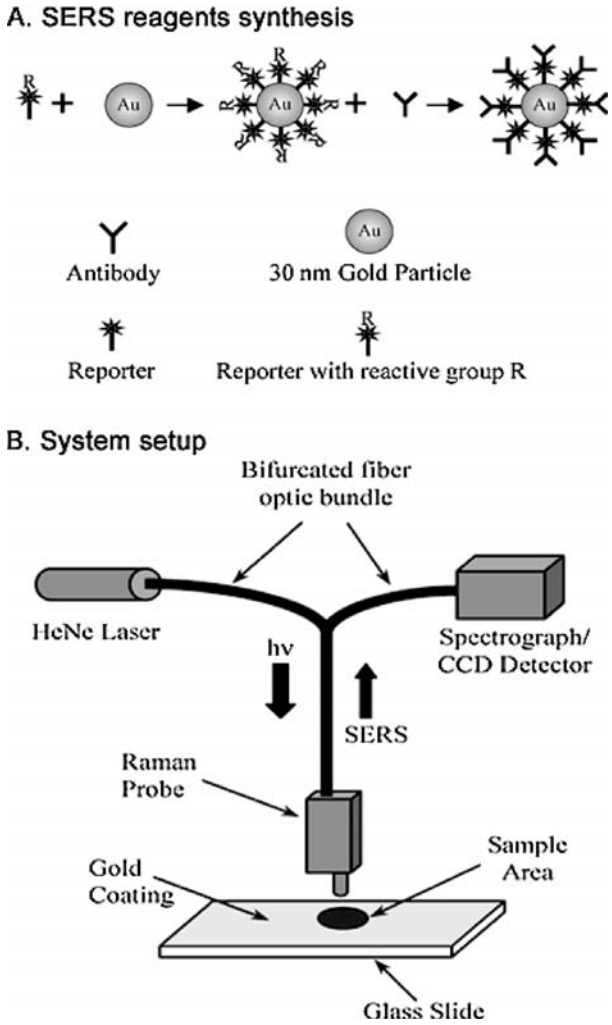

Figure 4. SERS reagents synthesis and experimental setup for measuring PSA levels in human serum using surface-enhanced Raman spectroscopy. Reprinted with permission from [15], D. S. Grubisha et al., *Anal. Chem.* 75, 5936 (2003). © 2003, American Chemical Society.

probe. The laser device is a 30-mW, 632.8-nm HeNe laser. The spectrograph has an f/2.0 Czerny–Turner imaging spectrometer plus a thermo-electrically cooled Kodak 0401 CCD camera. The fiberoptic probe uses band-pass and long-pass filters for laser light and fiber background rejection. For the detection of free PSA, its sensitivity was at low, 1 pg ml^{-1} (femtomolar level), in human serum [15], which is better than commercial assays based on radiometric, chemiluminescent, and ELISA methods (3–1000 pg ml^{-1}). This SERS-based surface is resistant to photobleaching and quenching. It has a narrow spectral bandwidth and long-wavelength excitation of multiple labels with a single excitation source, suitable for multianalyte assays. In addition, a so-called time-resolved immunofluorometric assay was recently created with nanoparticle-label technology, with a sensitivity for detection of free PSA at zeptomolar level (10^{-21} molar) in human serum samples [17–20].

The silicon nanowire field-effect device is another category of nanosensor for highly sensitive, label-free multiplexed electrical detection of biomarkers. Recently, an integrated nanowire array was developed, in which different

nanowires and surface receptors were incorporated as individual devices for the simultaneous detection of multiple protein markers at femtomolar levels [21]. There are three key steps in the fabrication of the device. Firstly, aldehyde propyltrimethoxysilane (APTMS) is coupled to oxygen plasma–cleaned silicon nanowire surfaces. This surface coating provides terminal aldehyde groups that are then coupled with monoclonal antibodies, while unbound groups are blocked by ethanolamine reaction. The basic nanowire sensor chip consists of integrated, electrically addressable silicon nanowires. These nanowires may be either p- or n-type. In addition, different receptors can be printed on the nanowire device to allow selective multiplex detection. Selective binding of the biomarkers to surface-coupled antibodies results in a specific conductance change in the corresponding receptor-modified silicon nanowire device, but not in the nanowire device lacking surface antibodies. With a p-type nanowire device, a well-defined conductance change is observed when delivering PSA-containing solution into the device. This change returns to baseline when a pure buffer is delivered. This reversibility indicates the specificity of the label-free protocol for direct measurement of biomarkers. PSA detection is routinely achieved with a signal-to-noise ratio >3 for concentrations down to 75 fg ml^{-1} (or 2 fM) of PSA.

In addition, multiplexed detection with two or more nanowire arrays can be used to discriminate possible electrical cross-talk and/or false-positive signals. For example, incorporation of p- and n-type nanowires in a single sensor chip can record simultaneous conductance-vs-time data by coupling different antibodies to the nanowires. Similar magnitudes of the conductance changes in the two devices indicate the true value of the target. Furthermore, this multiplexed sensor may be used to detect multiple biomarkers simultaneously by coupling different antibodies to the individual groups of nanowires.

Finally, these nanowire sensors can be used to measure biomarkers from human body fluids, e.g., serum. The sensitivity to human free PSA is 0.9 pg ml^{-1}, which corresponds to a concentration up to 100 billion times lower than that of the background serum proteins.

These nanowire arrays surpass previous biomarker detection systems using semiconductor nanowires and carbon nanotubes. The latter have 10^3–10^6 times lower target sensitivity, previous reports did not address their device-to-device reproducibility and selectivity, and no capacity of multiplexed detection was reported. The detection limits increased dramatically with nanowire arrays as compared to existing methods, including ELISA 3 pg ml^{-1} PSA. As mentioned earlier, sandwich-based magnetic and gold nanoparticles achieved two orders of magnitude better than nanowire arrays, but these assays require extensive labeling and multiple chemical and biochemical manipulations. Other label-free nanotechnologies using SPR (sensitivity 10–100 pg ml^{-1}) and microcantilevers (0.2 ng ml^{-1}) are 1000 times lower than nanowire assays, which also permit multiple targets detection for real patient samples.

It is hoped that new methods can differentiate between lethal and non-lethal cancers using proper biomarkers for prostate cancer. With the PSA assays discussed above, a prospective randomized long-term screening study is desirable to show improved survival in prostate cancer screening.

2. MOLECULAR IMAGING USING NANOTECHNIQUES FOR PROSTATE CANCER

In recent years, "personalized medicine," a novel concept, has become widely accepted as an ideal medical procedure [22], in which molecular imaging is the first requirement for it to become a reality. Molecular imaging has been defined in different ways; the most popular one is as follows: "The characterization and measurement of biologic processes in living animals, model systems, and humans at the cellular and molecular level by using remote imaging detectors" [23–24]. Therefore, a tracer that not only tracks a molecular process but also singles out a sign at acceptable resolution by a detecting device is critical. Generally, imaging tracers or probes can be grouped into three categories: non-specific, targeted, and activatable smart sensors [25]. An excellent example in prostate cancer is *ProstaScint*™ scan, a FDA-approved technique [26]. This technique uses a radiolabeled monoclonal antibody targeted to the intracellular domain of prostate-specific membrane antigen; the sensitivity and overall accuracy are summarized elsewhere [27].

Lymph-node metastasis is an adverse prognostic factor in prostate cancer and thus there is a critical need for identifying patients with this condition. Magnetic resonance imaging (MRI) usually provides excellent pictures with anatomical details [28]. The quality of the MRI picture can be further improved when contrast agents are used together, especially lymphotropic superparamagnetic manoparticles [29–31]. These nanoparticles are made of a magnetic iron oxide core and surface-coated with dextran for prolonged circulation time. They slowly extravasate from the vascular into the interstitial space, where they travel to and accumulate inside lymph nodes, where they are internalized by macrophages. These intracellular magnetic particles can increase the contrast on MRI images, which provides an excellent approach for non-invasive detection of primary tumors or of metastasis. As shown in Figure 5, MRI with lymphotropic nanoparticles correctly detected all lymph nodes with metastasis and had a significant higher sensitivity (71.4%, detailed in Table 2) over conventional MRI [30]. With the use of these nanoparticles, the signal-to-noise ratios of normal nodes dropped from 192 ± 24 (mean $\pm$ SD) to 69.7 ± 16 on T2-weighted sequences. On a patient-by-patient analysis, MRI with nanoparticles correctly identified all patients with metastasis (100% sensitivity). For those free of lymph node metastasis, the correct diagnosis was made 96% of the time. On a node-by-node basis, the overall sensitivity of MRI with the use of nanoparticles reached 90.5%, which was significantly higher than that of conventional MRI (34.5%). These data strongly suggest that magnetic nanoparticles greatly enhance the detection of mini-metastatic tumors in prostate cancers. This kind of enhancement is important and helpful, especially for decision-making, because patients with true localized tumors have the options of radical surgery, watchful waiting, or radiotherapy. For patients with locally advanced or metastatic disease, however, a much different therapeutic strategy will be put in place.

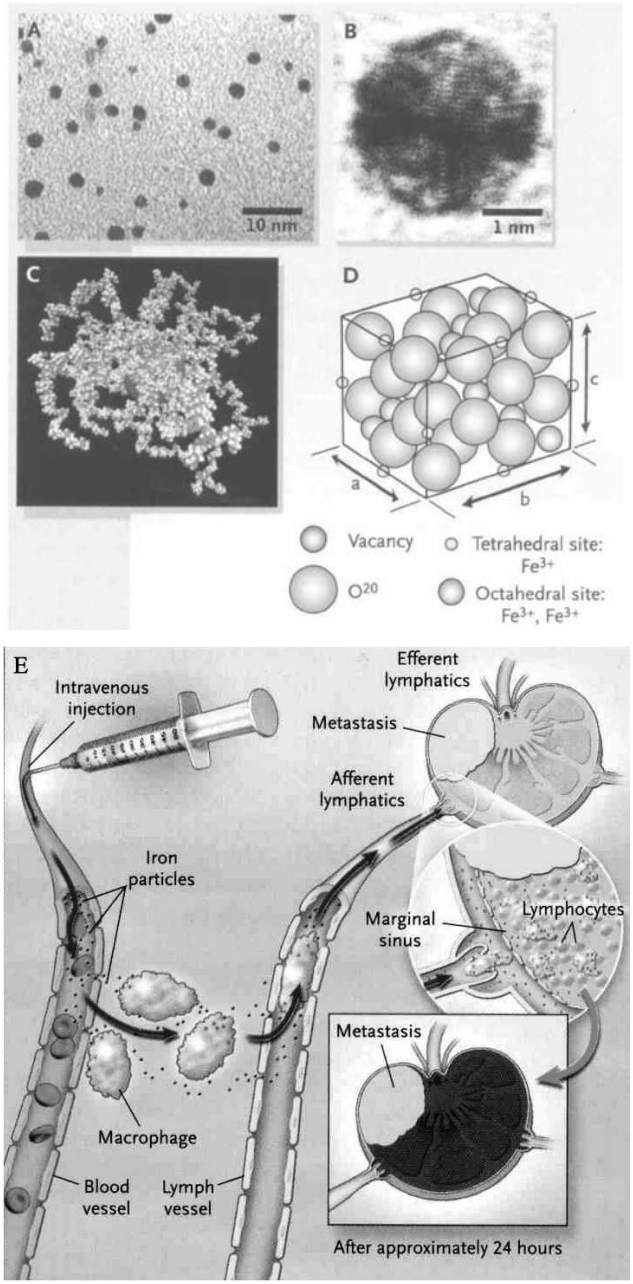

Figure 5. The model lymphotropic superparamagnetic nanoparticles measure 2–3 nm on average (A and B). The mean overall particle size of the 10 KD dextrans is 28 nm (C and D). **E.** The systemically injected long-circulating particles gain access to the interstitum and are drained through lymphatic vessels. Distributions in lymph flow or in nodal architecture caused by metastases lead to abnormal patterns of accumulation of lymphotropic superparamagnetic nanoparticles, which are detectable by MRI. Reprinted with permission from [30], M. G. Harisinghani et al., *N. Engl. J. Med.* 348, 2491 (2003). © 2003, Massachusetts Medical Society.

Quantum dots (QDs) are nanoscaled semiconductor particles that can be covalently conjugated with biorecognition molecules such as peptides, antibodies, nucleic acids, or small-molecule ligands. Compared to organic fluorophores, these QDs are fluorescence-tunable both in size and in composition from visible to infrared wavelengths, highly absorbable, brighter, and have long photostability [32–33]. There properties make QD as a perfect probe for targeted imaging at molecular level. A multifunctional QD probe for cancer cell targeting and imaging was reported and tested in prostate cancer model [34]. This QD has a core shell of CdSe-ZnS, which is coated with a coordinating ligand, tri-*n*-octylphosphine oxide (TOPO) and an amphiphilic polymer. These two coating materials interact tightly to form a hydrophobic protection structure that resists hydrolysis and enzymatic degradation *in vivo*. Based on the geometries, size constraints and the ligand coupling efficiencies, each QD contains up to 200 TOPO molecules, 4–5 triblock copolymers, 5–6 PEG and antibody molecules. After passive delivery into animals, the QDs accumulate preferentially at tumor sites through an enhanced permeability and retention effect. There are two hypotheses to explain this effect: (1) angiogenic tumors secret vascular endothelial growth factors that permeabilize the tumor-associated neovasculatures and result in a leakage of circulating macromolecules and small particles, and (2) most solid tumors lack an effective lymphatic drainage system, leading to macromolecule or nanoparticle accumulation. To actively deliver the QDs to prostate xenografts in nude mice, PSMA antibodies are conjugated onto the QD surface. Non-specific QD uptake was seen in liver and spleen only, but not in brain, heart, kidney, or lung after a single tail-vein injection of non-PMSA antibody conjugates. PMSA-conjugated QDs mainly accumulated in prostate cancer xenografts but not in liver or spleen. *In vivo* targeting experiments with human prostate cancer xenografts in mice demonstrated that as many as three million QD probes can be delivered into each cancer cell, measured by fluorescent intensity, without affecting cell viability and growth. QD-tagged cells showed a significantly stronger *in vivo* signal compared to the same level of GFP-tagged cells. Taken together, these polymer-encapsulated and bioconjugated QD probes are bright, stable, and have a versatile triblock copolymer structure that is highly suitable for *in vivo* multiplexed imaging, early detection, and targeted treatment of cancer patients. These QDs can be targeted to tumor sites through both passive and active approaches, but passive delivery is much slower and less efficient than active targeting. With a wavelength-resolved imaging system, the QDs can be detected sensitively and thus suitable for multiple color images of cancer cells in living animals.

3. NANOPARTICLE-BASED TARGETING DELIVERY FOR PROSTATE CANCER THERAPY

Targeted delivery of therapeutic reagents for solid tumors has been a major challenge for many years in nanotechnology. There are four major needles for those therapeutic nanoparticles to be functional right on directly the tumor cells: escaping from the reticuloendothelial capturing system (RES), passing through the blood vessel endothelial barrier, diffusing within the extracellular matrix, and then penetrating the cellular plasma membrane. Successful strategies have been developed to help the nanoparticles in evade RES capture, including the Stealth™ liposome [35] and polymerized nanotechnologies [36–37]. These techniques have been

Table 2. Sensitivity, specificity, accuracy, and positive and negative predictive values of MRI alone and MRI with lymphotropic superparamagnetic nanoparticles.

Variable	MRI alone	MRI with lymphotropic superparamagnetic nanoparticles	P value
Results per patient ($n = 80$)			
Sensitivity (%)	45.4	100	<0.001
Specificity (%)	78.7	95.7	
Accuracy (%)	65.0	97.5	
Positive predictive value (%)	60.0	94.2	
Negative predictive value (%)	67.2	100	
Results per individual lymph nodes of all sizes ($n = 334$)			
Sensitivity (%)	35.4	90.5	<0.001
Specificity (%)	90.4	97.8	
Accuracy (%)	76.3	97.3	
Positive predictive value (%)	55.9	95.0	
Negative predictive value (%)	80.3	97.8	
Area under the curve	0.756	0.975	<0.001
Results for nodes with a short-axis diameter of 5–10 mm ($n = 45$)			
Sensitivity (%)	28.5	96.4	<0.001
Specificity (%)	87.2	99.3	
Accuracy (%)	78.3	98.9	
Positive predictive value (%)	28.5	96.4	
Negative predictive value (%)	87.2	99.3	
Results for nodes with a short-axis diameter of <5 mm ($n = 17$)			
Sensitivity (%)	0	41.1	
Specificity (%)	100	98.1	
Accuracy (%)	86.4	90.4	
Positive predictive value (%)	NA*	77.7	
Negative predictive value (%)	86.4	91.3	

Source: Reprinted with permission from [30], M. G. Harisinghani et al., *N. Engl. J. Med.* 348, 2491 (2003). © 2003, Massachusetts Medical Society.

*NA denotes are not applicable.

extensively reviewed [1, 23, 38–41], and several examples in prostate cancer model are summarized below.

Since viral vectors have toxicity and immunogenicity, non-viral vector systems have been investigated for gene delivery [42]. Biodegradable polymeric nanoparticles with entrapped plasmid DNA or chemotherapeutic drugs is considered to be the most promising of those non-viral vehicles [43]. In comparison to matrix-type polymer-based nanoparticles, poly(D,L-lactide-*co*-glycolide) (PLGA) and polylactide (PLA)-based nanoparticles are especially of interest for gene delivery because of their biocompatibility, biodegradability, and sustained release characteristics.

Cellular uptake of the nanoparticles is through an energy-dependent endocytic process. The uptake of nanoparticles decreases in the presence of metabolic inhibitors (sodium azide and deoxyglucose), confirming the endocytic process of uptake of nanoparticles. PLGA-nanoparticles are rapidly released from the endolysosomes into the cytoplasm. Nanoparticles that escaped the endosomes into the cytoplasmic compartment slowly released the entrapped DNA, resulting in sustained gene expression. Factors that affect the intracellular uptake of nanoparticles and distribution, or DNA loading and release will be the main determinants for nanoparticle-mediated gene transfection. Using human prostate cancer PC-3 as a model, PLGA polymer-based nanoparticles showed higher gene transfection (due to higher DNA release) than those formulated using PLA polymer. More specifically, nanoparticles made from higher-molecular-weight PLGA carried more DNA loading, which resulted in higher gene expression than those formulated with lower-molecular-weight PLGA. Higher-molecular-weight (143 kD) polymer forms smaller nanoparticles, more uniform in shape and size, relatively lower negative ζ potentials, and higher DNA release rate *in vitro* and *in vivo* as compared to those formulated using low-molecular-weight polymers.

Nanoparticle surface characteristics are also critical determinants. PVA is a commonly used emulsifier in the formulation of nanoparticles. After formulation, a fraction of PVA remains associated with PLGA nanoparticles. The remaining PVA was found to influence the efficiency of PLGA nanoparticles. For example, nanoparticles with lower amounts of surface-associated PVA showed higher gene transfection rates, which is due to their higher intracellular uptake and cytoplasmic levels. Therefore, DNA loading in nanoparticles and its release, and surface-associated PVA are the critical determinants in nanoparticle-mediated gene transfection [44].

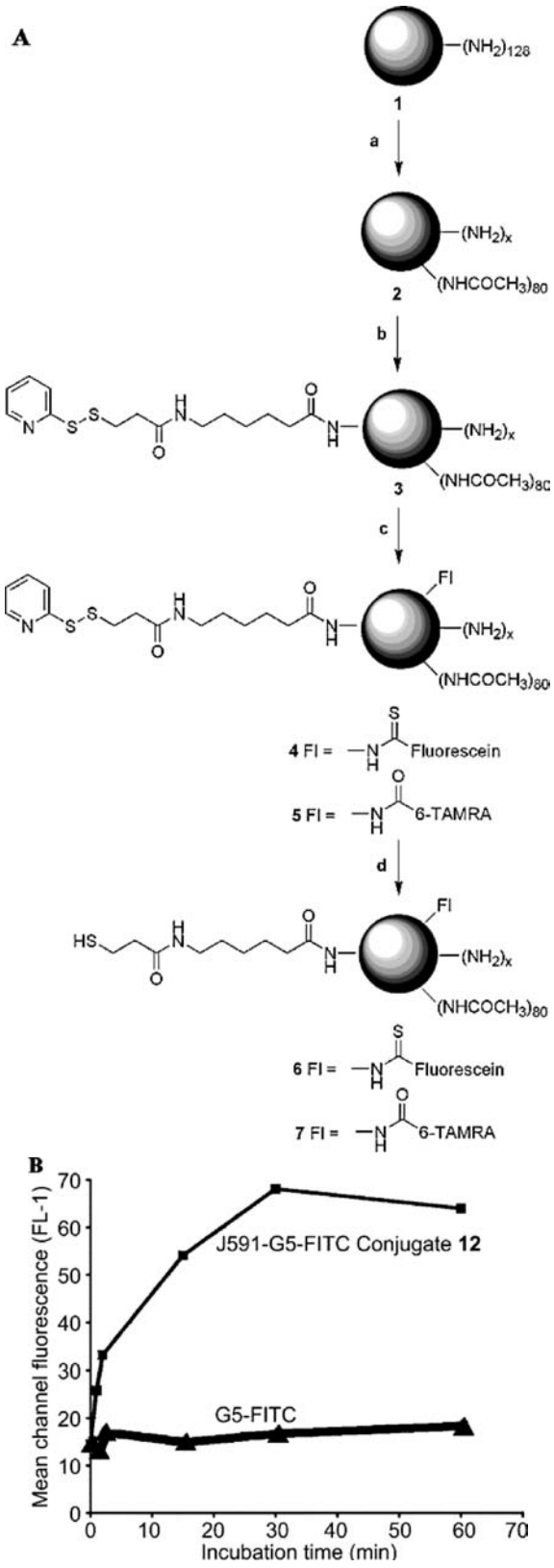

Figure 6. A, Synthesis of Dendrimer Conjugates by Terminal Group Modifications on Generation 5 PAMAM Dendrimer. Reagents and conditions: (a) Acetic anhydride, Et_3N, methanol, rt, 16 h; (b) Sulfo-LC-SPDP, PBS, rt, 3 h; (c) FITC or 6-TAMRA SE, DMSO; (d) DTT, PBS-EDTA. B, Kinetics of binding J591-G5-FITC dendrimer to LNCaP.FGC cells. The cells were incubated with a 50 nM concentration of 12 or control G5-FITC dendrimer (**9a**) for the indicated periods of time at rt. After incubation, cells were washed and the mean channel fluorescence of stained cells was determined by FCM. Reprinted with permission from [47], A. K. Patri et al., *Bioconjug. Chem.* 15, 1174 (2004). © 2004, American Chemical Society.

Unlike polymers that have a range of molecular weight distribution, novel branched dendrimers have a defined molecular weight and more controllable peripheral functionalities [45]. The first dendrimers used for gene transfer was polyamidoamine (PAMAM). Although modification of the terminal groups with different aromatic and hydrophobic molecules allows delivery of various kinds of drugs, the PAMAM conjugates lose aqueous solubility. Acetylation of those modified terminal groups will reduce aggregate formation and decrease nonspecific binding to the cell surface. A recent report showed that synthesized anti-PSMA antibody J591–dendrimer conjugates specifically bind to cells that express PSMA, as shown in Figure 6 [46]. Non-antibody–conjugated PAMAM dendrimers do not show any significant binding. Confocal microscopy experiments demonstrated the internalization of the anti-PSMA antibody-conjugated PAMAM dendrimers in the PSMA-expressing human prostate cancer LNCaP cells. These results encourage the further study of antibody–dendrimer conjugates for targeted therapeutics.

Polypropylenimines (PPI) are similar to PAMAM, containing terminal amino groups. Repetitively adding a primary amine to two equivalents of acrylonitile and then catalytically hydrogenating it can produce polyamine-terminated PPI dendrimers. Currently, five generations of PPI dendrimers have been produced. Dendrimers made from PPI were tested for delivering a 31-nt triplex-forming oligonucleotide (ODN) in prostate cancer LNCaP cell lines, using a ^{32}P-labeled ODN [47]. The dendrimers did not have significant effect on cell viability. In comparison to control ODN uptake, dendrimer-mediated uptake of ODN was higher by 14-fold. Among various dendrimers, generation 4 (G-4) dendrimer has the maximum efficacy in a concentration- and molecular weight–dependent manner. Complexing ODN with G-4 dendrimers significantly increased the growth-inhibitory effect of the ODN, which remained intact in cells even after 48 h of treatment. Therefore, polypropylenimine dendrimers might be useful vehicles for delivering therapeutic oligonucleotides to cancer cells.

The vitamin folic acid (FA) is a high-affinity ligand for folate receptor (FR), and is used as a targeting molecule for the delivery of FR-conjugated therapeutic drugs to cancer tissue [48]. FR is overexpressed in multiple organ or tissue types including breast, ovary, endometrium, kidney, lung, head, neck, brain, and myeloid cancers and is internalized into cells after ligand binding [49–52]. In human prostate, there is high-affinity folate-binding activity [53], indicating the possible use of folate-linked nanoparticles as a potential vehicle for prostate cancer treatment. A group from Japan developed a folate-linked nanoparticle (NP-F) and evaluated its potential in targeted gene delivery and efficiency in *in vitro* cell-based assay and in *in vivo* mice xenograft

Figure 6. Continued.

models [54–55]. These NP-F particles consist of 3([*N*-(*N*′, *N*′-dimethylaminenoethane)-carbonamoyl] cholesterol (DC-Chol), Tween-80, and folate-polyethylenglycol-distearoyl-phosphatidyl-ethanolamine conjugate (f-PEG-DSPE). Six different cationic NPs were formulated by varying these components. The average sizes of the NPs were about 100–200 nm and their ζ-potentials were about +50 mV. After mixing with DNA, NP size increased slightly from 200 nm to 300 nm, and the ζ-potential decreased to +30–40 mV. However, folate-conjugated NPs did not change significantly after mixing with water, serum, or DNA. In human prostate cancer LNCaP and PC-3 cells, as shown in Figure 7, a high transfection efficiency was achieved when the NP-F was mixed with plasmid DNA carrying herpes simplex virus thymidine kinase (HSV-tk) and connexin 43 (C × 43). Cell death was significantly induced in the presence of the prodrug ganciclovir (GCV). The GCV sensitivity increased significantly in LNCaP cells transfected with pCMV-tk compared with the control (85.6-fold increase), or in LNCaP cells transfected with pSV40-C × 43 compared with the control (6.6-fold increase), and in LNCaP cells co-transfected with both plasmids compared with the control (101.8-fold increase). In the xenograft model, intratumoral injection of the NP-F mixed with either HSV-tk gene alone or HSV-tk plus C × 43 genes, together with intraperitoneal administration of GCV, dramatically suppressed tumor growth. The survival time was significantly prolonged in the treatment group (33 days, compared with the control group (21.5 days)). This work provides strong support for clinical testing of folate-based tumor targeting strategies.

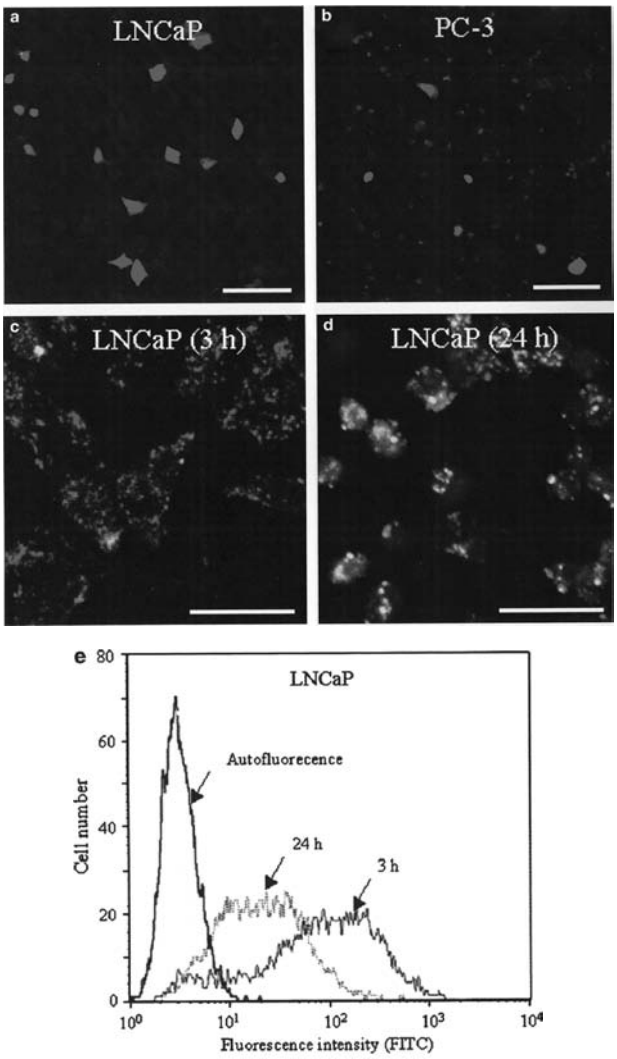

Figure 7. Uptake and expression of DNA with NP-F nanoplex. NP-F mixed with the pEGFP-C1 was incubated with (a) LNCaP and (b) PC-3 cells for 24 h. Dil-labeled NP-F mixed with FITC-ODN was incubated with LNCaP for (c) 3 h or (d) 24 h. GFP expression, Dil-labeled NP-F or FITC-ODN was visualized by confocal microscopy. In (e), the association of DNA with NP-F was determined based on FITC-ODN by flow cytometry. Reprinted with permission from [55], Y. Hattori and Y. Maitani, *Cancer Gene Ther.* 12, 796 (2005). © 2004, Nature Publishing Group.

Transferrin (Tf) is another ligand that has been extensively studied for tumor-targeting, since transferrin receptor is overexpressed in tumor cells compared with normal cells [56]. Human Tf-targeted cationic liposome–DNA complex (Tf-lipoplex) has a high efficiency in gene delivery in cancer cell models, including prostate cancers [57–58]. Tf-lipoplex has a highly compact structure, and its size is relatively uniform (50–90 nm). It resembles a virus particle with a dense core enveloped by a membrane coated with Tf molecules. Compared with lipoplex without Tf ligand, Tf-lipoplex is more stable, and its *in vivo* gene transfer efficiency is much higher. When used together with radiotherapy, its long-term efficacy for systemic p53 gene delivery is higher than unliganded lipoplex in human prostate cancer (Fig. 8). Another report also tested if Tf-conjugation significantly enhances the efficiency of paclitaxel (Tx)-loaded

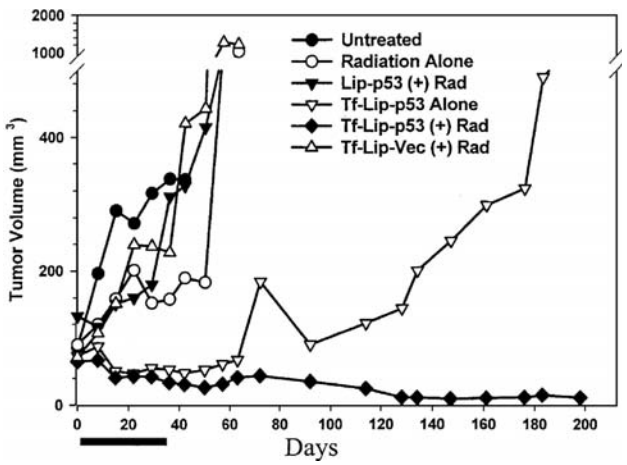

Figure 8. Effect of the combination of Tf-Lip-p53 and radiation treatment on DU-145 xenograft *in vivo*. Nude mice bearing tumor of ~70 mm^3 in volume were injected, via tail vein, with Tf-Lip-p53, Tf-Lip-pVed (plasmid vector without p53), or non-targeted Lip-p53, 10 μg of DNA per 0.3 ml per injection, twice weekly for a total of 10 injections, and the tumors only were exposed to 2.0 Gy fractioned doses of γ radiation (total, 40 Gy). There were five nude mice per group. The horizontal bar indicates the duration of treatment. The Tf-Lip-p53 plus radiation-treated group exhibited completed tumor regression and showed no sign of recurrence even 6 months after treatment. Reprinted with permission from [58], L. Xu et al., *Hum. Gene Ther.* 13, 469 (2002). © 2002, Mary Ann Liebert, Inc.

biodegradable nanoparticles (NPs) on tumor inhibition [59]. The anti-tumor activity of NPs was assessed in human prostate cancer cell line PC3 *in vitro* and *in vivo*. The NPs are approximately 220 nm in diameter, and its drug loading is about 5.4% w/w. Under *in vitro* conditions its release rate of the encapsulated drug is 60% in 60 days. The amount of Tf conjugated to NPs was 29 μg ml^{-1} NPs, which represents about 440 Tf molecules per NP. The uptake of Tf-conjugated NPs was increased about 3-fold compared with unconjugated NPs in human prostate cancer cell line PC-3. This increased uptake was inhibited if the was excessive free Tf in the medium. A 10-fold higher dosage of the drug in solution (10 ng ml^{-1}) was required to achieve a similar effect as that with Tx-NPs-Tf. The IC-50 of Tf-conjugated NPs (Tx-NPs-Tf) showed a 5-fold increase over unconjugated NPs (Tx-NPs) or and over drug in solution, in terms of tumor cell growth. Animal experiments showed that a single-dose intratumoral injection of Tx-NPs-Tf resulted in complete tumor regression and a greater survival rate than those that received either Tx-NPs or Tx-Cremophor EL formulation. With 24 mg kg^{-1} drug dose, a complete regression of tumor growth was observed. The survival rate in the Tx-NPs-Tf treatment group was significantly higher compared with the control group. These studies demonstrated that Tf-liganded NPs has greater gene/drug delivery efficiency.

In addition to antibodies and peptides, nucleic acids have also tested as ligands for targeted delivery, so-called aptamers [60–61]. Aptamers are RNA or DNA oligonucleotides. These oligonucleotides fold into unique 3-D structures and bind to cellular targets by complementary shape interactions with high affinity and specificity. Aptamers are not immunogenic or toxic, are small in size, and are easy to handle. All of these have resulted in rapid progress of this field. Recently, a fully automatic system, systemic evolution of ligands by exponential enrichment (so-called SELEX), was established to identify target specific aptamers. This unique procedure reduces the *in vitro* selection time from months to days [62].

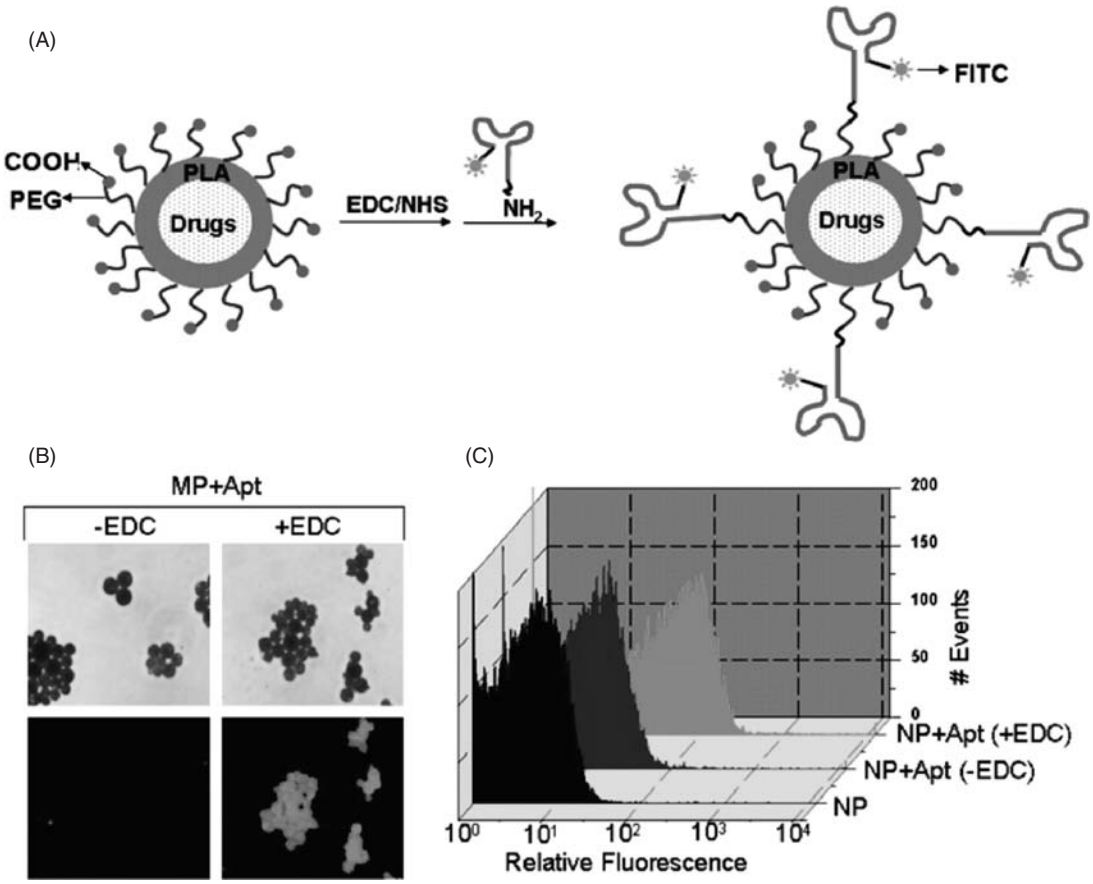

Figure 9. A. Schematic diagram of the conjugation reaction of PLA-PEG-COOH nanoparticles or microparticles with 3′-NH_2-modified, 5′-FITC-labeled PSMA aptamers. B. Conjugation of microparticles with aptamers. The acid group on the surface of PLA-PEG-COOH microparticles (MP) were left untreated (−EDC) or converted to NHS ester (+EDC) and particles were incubated with NH_2-modified FITC-labeled PSMA aptamers. After the wash, the microparticles resulting after nonspecific interaction with aptamers (−EDC) or after covalent linkage with aptamers (+EDC) were visualized by (*top row*) transmission light microscopy and (*bottom row*) fluorescent microscopy. C. Conjugation of nanoparticles with aptamers. The acid group on the surface of PLA-PEG-COOH nanoparticles were left untreated (−EDC) or converted to NHS ester (+EDC), and particles were examined without aptamers (NP, *black curve*) or incubated with NH_2-modified FITC-labeled aptamers (+Apt). The bioconjugates resulting from the covalent linkage of aptamers and nanoparticles [NP + Apt (+EDC, *green curve*)] demonstrated an approximately 7-fold increase in fluorescence when compared with nanoparticles that were incubated but had no covalent linkage to aptamers [NP + Apt (−EDC, *blue curve*)]. Reprinted with permission from [63], O. C. Farokhzad et al., *Cancer Res.* 64, 7668 (2004). © 2004, American Association for Cancer Research.

SELEX usually takes about 5 to 20 iterative rounds of selection and application. Selective aptamers can be targeted to any molecules, including those agents that are impossible to raise antibodies or vaccines against. Once the nucleotide sequences are defined, they can be produced easily *in vitro* by enzymatic or chemical synthesis. They can be also customized by modifying certain positions to increase half-lives, to conjugate with imaging probes, and to distribute to particular tissue/cell locations. Their binding affinities are comparable to monoclonal antibodies. Currently, several commercialized automatic SELEX platforms are available for high-throughput screening assays [62].

For prostate cancer, the first report of targeted drug delivery with nanoparticle–aptamer bioconjugates was reported last year [63–64]. It was an RNA aptamer that targets human prostate-specific membrane antigen (PSMA) and was conjugated with a polymer-based nanoparticle (Fig. 9). This nanoparticle was taken up efficiently by prostate LNCaP epithelial cells. In contrast to LNCaP cells, the uptake of these particles is not enhanced in cells that do not express the prostate-specific membrane antigen protein.

In combination with an external localized magnetic field gradient applied to a selective organ site, magnetic nanoparticles may provide an ultimate opportunity for targeted drug delivery for cancer patients [65]. As a drug delivery vehicle, magnetic nanoparticles should have a good drug-loading capacity, desirable drug release profile, aqueous dispersion stability, biocompatibility with human tissues and organs, and reliable retention after modification with polymers or chemical reaction. Recently, a novel oleic acid (OA)-Pluronic-stabilized iron oxide magnetic nanoparticle was formulated as a drug carrier for anti-tumor reagents. The shell formed by OA-coating on the iron oxide nanoparticle surface encapsulates hydrophobic drugs and Pluronic anchored to the OA interface ensures the dispersity of the drug-loaded nanoparticles. Iron oxide surface is hydrophobic; therefore, it is not dispersible in nonpolar solvents such as hexane and chloroform. After OA coating, the surface becomes hydrophilic; thus the nanoparticle becomes dispersible in nonpolar solvents. Complete coating of the iron oxide nanoparticle surface is required to achieve a total dispersion. The optimized composition for the iron oxide nanoparticle was 70.1 wt % iron oxide, 15.4 wt % OA, and 14.5 wt % Pluronic. The nominal composition was 63 wt % iron oxide, 18.3 wt % OA, and 18.7 wt % Pluronic. As a test model, anti-cancer drug doxirubicin (DOX) was used to evaluate the drug-loading capacity of this nanoparticle formulation. DOX loading was 8.2 ± 0.5 wt % (i.e., 82 μg of drug mg^{-1} of nanoparticles) with an encapsulation efficiency of 82%. Drug release profile was sustained, with about 28% cumulative release in 2 days and 62% within one week. Cytotoxicity experiments showed that no toxic effect was observed when an empty nanoparticle was used. DOX-loaded nanoparticles showed a significant dose-dependent cytotoxic effect upon its exposure to human prostate cancer PC-3 cells. Confocal imaging microscopy demonstrated that the drug was localized in the nucleus after 24–48 h post-nanoparticle treatment [65].

In conclusion, we have summarized here current works using nanotechniques in prostate cancer management. Novel diagnostic and prognostic biomarkers are needed for prostate cancer, and the ultimate goal is to find accurate biomarkers for early diagnosis and identification of patients at greatest risk of developing aggressive disease. Nanotechnology is being brought to bear on this problem; however, one limitation is that not much work so far has been done for prostate cancers. There is a great deal more that should be done to develop novel strategies for the prevention and treatment of prostate cancers using those nano-scaled techniques.

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